FORM PTO-1390 (REV. 9-2001) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: REG 710-A-US

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): NOT KNOWN 10/009852

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/14142	May 23, 2000	June 8, 1999

TITLE OF INVENTION: MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES AND METHODS OF MAKING AND USING THEREOF

APPLICANT(S) FOR DO/EO/US: Nicholas J. Papadopoulos, Samuel Davis, and George D. Yancopoulos

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [X] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [X] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [X] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. [X] Other items or information: Express Mail Label No. ET712522493US dated December 6, 2001

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
Not Yet Known 10/009852	PCT/US00/14142	REG 710-A-US

21. [X] The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO $1040.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO $740.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) $710.00		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 740.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	154 - 20 =	134	x $18.00	$ 2,412.	
Independent claims	5 - 3 =	2	x $84.00	$ 168.	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $280.00	$ 280.	
TOTAL OF ABOVE CALCULATIONS =				$ 3,600	
[ ] Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$	
SUBTOTAL =				$ 3,600.	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 3,600.	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ 3,600.	
				Amount to be refunded:	$
				charged:	$

a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 18-0650 in the amount of $ 3,600. to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-0650 . A duplicate copy of this sheet is enclosed.

d. [ ] Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Linda O. Palladino
Patent Agent
Regeneron Pharmaceuticals, Inc.
777 Old Saw Mill River Road
Tarrytown, New York 10591

Linda O. Palladino
SIGNATURE 12/6/01

Linda O. Palladino
NAME

45,636
REGISTRATION NUMBER

Att. Docket No. REG 710-A-US

FIRST CLASS MAIL CERTIFICATE
I hereby certify that this document is being deposited with the United States Postal Service on this date as first class mail addressed to: Commissioner for Patents, U.S. Patent and Trademark Office, Washington, D.C. 20231.

Linda O. Palladino — December 6, 2001
Linda O. Palladino — Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application Of	:	Nicholas J. Papadopoulos, Samuel Davis, and George D. Yancopoulos
USSN	:	Not Yet Known
Filed	:	Filed Herewith
Int'l File No.	:	PCT/US00/14142
Int'l File Date	:	May 23, 2000
For	:	MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES AND METHODS OF MAKING AND USING THEREOF
		December 6, 2001

Commissioner for Patents
U.S. Patent and Trademark Office
Washington, D.C. 20231

Att:

PRELIMINARY AMENDMENT

Sir:

This paper is submitted in connection with the above-identified application. Prior to examination of the application on the merits, please amend the specification as follows:

In the Claims:

Please replace Claim 9, starting on page 92, line 20, through page 93, line 10 with the following:

9. (Amended) An isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in Figure 13A-13D (SEQ ID NOS: 3 and 4);

(b) the nucleotide sequence set forth in Figure 14A-14C (SEQ ID NOS: 5 and 6);

(c) the nucleotide sequence set forth in Figure 15A-15C (SEQ ID NOS: 7 and 8);

(d) the nucleotide sequence set forth in Figure 16A-16D (SEQ ID NOS: 9 and 10);

(e) the nucleotide sequence set forth in Figure 21A-21C (SEQ ID NOS: 11 and 12);

(f) the nucleotide sequence set forth in Figure 22A-22C (SEQ ID NOS: 13 and 14);

(g) the nucleotide sequence set forth in Figure 24A-24C (SEQ ID NOS: 15 and 16); and

(h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c),

(d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having the biological activity of the modified Flt1 receptor fusion polypeptide.

Please replace Claim 22, starting on page 95, line 1, with the following:

22. (Amended) A fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D (SEQ ID NOS: 1 and 2) or Figure 24A-24C (SEQ ID NOS: 15 and 16), which has been modified by acetylation or pegylation.

Please replace Claim 49, starting on page 98, line 24, through page 99, line 8, with the following:

49. (Amended) An fusion polypeptide comprising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from the group consisting of:

(a) the amino acid sequence set forth in Figure 13A-13D (SEQ ID NOS: 3 and 4);

(b) the amino acid sequence set forth in Figure 14A-14C (SEQ ID NOS: 5 and 6);

(c) the amino acid sequence set forth in Figure 15A-15C (SEQ ID NOS: 7 and 8);

(d) the amino acid sequence set forth in Figure 16A-16D (SEQ ID NOS: 9 and 10);

(e) the amino acid sequence set forth in Figure 21A-21C (SEQ ID NOS: 11 and 12)

(f) the amino acid sequence set forth in Figure 22A-22C (SEQ ID NOS: 13 and 14); and

(g) the amino acid sequence set forth in Figure 24A-24C (SEQ ID NOS: 15 and 16).

In the Specification:

Please replace the paragraph starting on page 1, line 5, with the following:

The application claims priority of International Application No. PCT/US00/14142, filed May 23, 2000, which claims priority of U.S. Provisional Application No. 60/138,133, filed on June 8, 1999. Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

Please replace the paragraph starting on page 11, line 15, through page 12, line 1, with the following:

Preferred embodiments include an isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of (a) the nucleotide sequence set forth in Figure 13A-13D (SEQ ID NOS; 3 and 4);

(b) the nucleotide sequence set forth in Figure 14A-14C (SEQ ID NOS: 5 and 6);

(c) the nucleotide sequence set forth in Figure 15A-15C (SEQ ID NOS: 7 and 8);

(d) the nucleotide sequence set forth in Figure 16A-16D (SEQ ID NOS: 9 and 10);

(e) the nucleotide sequence set forth in Figure 21A-21C (SEQ ID NOS: 11 and 12);

(f) the nucleotide sequence set forth in Figure 22A-22C (SEQ ID NOS: 13 and 14);

(g) the nucleotide sequence set forth in Figure 24A-24C (SEQ ID NOS: 15 and 16); and

(h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c), (d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having the biological activity of the modified Flt1 receptor fusion polypeptide.

Please replace the paragraph starting on page 13, line 6, with the following:

Additional embodiments include a fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D (SEQ ID NOS: 1 and 2) or Figure 24A-24C (SEQ ID NOS: 15 and 16), which has been modified by acetylation or pegylation wherein the acetylation is accomplished with at least about a 100 fold molar excess of acetylation reagent or wherein

acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess or wherein the pegylation is 10K or 20K PEG.

Please replace the paragraph starting on page 15, line 19, with the following:

Preferred embodiments include a fusion polypeptide comprising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from the group consisting of (a) the amino acid sequence set forth in Figure 13A-13D (SEQ ID NOS: 3 and 4); (b) the amino acid sequence set forth in Figure 14A-14C (SEQ ID NOS: 5 and 6); (c) the amino acid sequence set forth in Figure 15A-15C (SEQ ID NOS: 7 and 8); (d) the amino acid sequence set forth in Figure 16A-16D (SEQ ID NOS 9 and 10); (e) the amino acid sequence set forth in Figure 21A-21C (SEQ ID NOS: 11 and 12); (f) the amino acid sequence set forth in Figure 22A-22C (SEQ ID NOS: 13 and 14) and (g) the amino acid sequence set forth in Figure 24A-24C (SEQ ID NOS: 15 and 16).

Please replace the paragraph starting on page 19, line 11, with the following:

Figure 10A-10D (SEQ ID NOS: 1 and 2). Nucleic acid (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) of Flt1(1-3)-Fc.

Please replace the paragraph starting on page 19, line 16, with the following:

Figure 13A-13D (SEQ ID NOS: 3 and 4). Nucleic acid (SEQ ID NO: 3) and deduced amino acid sequence (SEQ ID NO: 4) of Mut1: Flt1(1-3$_{\Delta B}$)-Fc.

Please replace the paragraph starting on page 19, line 22, with the following:

Figure 14A-14 C (SEQ ID NOS: 5 and 6). Nucleic acid (SEQ ID NO: 5) and deduced amino acid sequence (SEQ ID NO: 6) of Mut2: Flt1(2-3$_{\Delta B}$)-Fc.

Please replace the paragraph starting on page 19, line 25, with the following:

Figure 15A-15C (SEQ ID NOS: 7 and 8). Nucleic acid (SEQ ID NO: 7) and deduced amino acid sequence (SEQ ID NO: 8) of Mut3: Flt1(2-3)-Fc.

Please replace the paragraph starting on page 20, line 1 with the following:

Figure 16A-16D (SEQ ID NOS. 9 and 10). Nucleic acid (SEQ ID NO: 9) and deduced amino acid sequence (SEQ ID NO: 10) of Mut4: Flt1(1-3$_{R->N}$)-Fc.

Please replace the paragraph starting on page 21, line 16, with the following:

Figure 21A-21C (SEQ ID NOS: 11 and 12). Nucleotide (SEQ ID NO: 11) and deduced amino acid sequence (SEQ ID NO: 12) of the modified Flt1 receptor termed Flt1D2.Flk1D3.FcΔC1(a).

Please replace the paragraph starting on page 21, line 19, with the following:

Figure 22A-22C (SEQ ID NOS: 13 and 14). Nucleotide (SEQ ID NO: 13) and deduced amino acid sequence (SEQ ID NO: 14) of the modified Flt1 receptor termed Flt1D2.VEGFR3D3.FcΔC1(a).

Please replace the paragraph starting on page 22, line 1, with the following:

Figure 24A-24C (SEQ ID NOS: 15 and 16). Nucleotide (SEQ ID NO: 15) and deduced amino acid sequence (SEQ ID NO: 16) of the modified Flt1 receptor termed VEGFR1R2-FcΔC1(a).

Please replace the paragraph starting on page 25, line 18, with the following:

Figure 36 (SEQ ID NO: 17). Peptide mapping and glycosylation analysis. The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 is disulfide bonded to Cys76. Cys121 is disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, it can not be determined whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is disulfide bonded to Cys306. Cys 352 is disulfide bonded to Cys410.

Please replace the paragraph starting on page 49, line 25, through page 51, line 12, with the following:

Based on the observation that acetylated Flt1(1-3)-Fc, which has a pI below 6, has much better pharmacokinetics than the highly positive unmodified Flt1(1-3)-Fc (pI > 9.3), it was asked whether the difference in pharmacokinetics could be attributed to the net charge of the protein, which made it stick to negatively charged extracellular matrix components, or whether there were perhaps specific locations on the surface of the Flt1(1-3)-Fc protein that constituted specific binding sites for extracellular matrix components. For example, many proteins are known to have heparin binding sites, often consisting of a cluster of basic residues. Sometimes these residues are found in a cluster on the primary sequence of the protein; some of the literature has identified "consensus

sequences" for such heparin binding sites (see for example Hileman, et al., 1998, Bioessays 20(2):156-67). In other cases, the known crystal structure of a protein reveals a cluster of positively charged residues on the surface of a protein, but the residues come from different regions of the primary sequence and are only brought together when the protein folds into its tertiary structure. Thus it is difficult to deduce whether an isolated amino acid residue forms part of a cluster of basic residues on the surface of the protein. However, if there is a cluster of positively charged amino acid residues in the primary sequence, it is not unreasonable to surmise that the residues are spatially close to one another and might therefore be part of an extracellular matrix component binding site. Flt1 receptor has been studied extensively and various domains have been described (see for example Tanaka et al., 1997, Jpn. J. Cancer Res 88:867-876). Referring to the nucleic acid and amino acid sequence set forth in Figure 10A-10D (SEQ ID NOS: 1 and 2) of this application, one can identify the signal sequence for secretion which is located at the beginning of the sequence and extends to the glycine coded for by nucleotides 76-78. The mature protein begins with Ser-Lys-Leu-Lys (SEQ ID NO: 35), starting at nucleotide 79 of the nucleic acid sequence. Flt1 Ig domain 1 extends from nucleotide 79 to 393, ending with the amino acids Ser-Asp-Thr. Flt1 Ig domain 2 extends from nucleotide 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1 Ig domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val to Asp-Lys-Ala). There is a bridging amino acid sequence, Gly-Pro-Gly, encoded by nucleotides 997-1005, followed by the nucleotide sequence

encoding human Fc (nucleotides 1006-1701 or amino acids Glu-Pro-Lys to Pro-Gly-Lys-stop).

Please replace the paragraph starting on page 51, line 14, through page 52, line 16, with the following:

A more detailed analysis of the Flt1 amino acid sequence reveals that there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR [SEQ ID NO: 36]) of Figure 10A-10D (SEQ ID NOS: 1 and 2), in which 6 out of 10 amino acid residues are basic. This sequence is located in Flt1 Ig domain 3 of the receptor (see Figure 11), which is not itself essential for binding of VEGF ligand, but which confers a higher affinity binding to ligand. An alignment of the sequence of Ig domain 3 with that of Ig domain 2 reveals that in this region, there is very poor alignment between the two Ig domains, and that there are about 10 additional amino acids in Ig domain 3. An analysis of the hydrophilicity profiles (MacVector computer software) of these two domains clearly indicates the presence of a hydrophilic region in the protein (Figure 12A-12B). These observations raised the possibility that the actual three dimensional conformation of Flt1 Ig domain 3 allowed for some type of protrusion that is not in Flt1 Ig domain 2. To test this hypothesis, the 10 additional amino acids were deleted and the resulting protein was tested to see whether the deletion would affect the pharmacokinetics favorably without seriously compromising the affinity of the receptor for VEGF. This DNA construct, which was constructed using standard molecular biology techniques (see

e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), is referred to as Mut1: Flt1(1-3$_{\Delta B}$)-Fc. The Mut1: Flt1(1-3$_{\Delta B}$)-Fc construct was derived from Flt1(1-3)-Fc by deletion of nucleotides 814-843 (set forth in Figure 10A-10D [SEQ ID NOS: 1 and 2]), which deletes the highly basic 10-amino acid residue sequence Lys-Asn-Lys-Arg-Ala-Ser-Val-Arg-Arg-Arg (SEQ ID NO: 32) from Flt1 Ig domain 3.

Please replace the paragraph starting on page 52, line 18, with the following:

The final DNA construct was sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut1: Flt1(1-3$_{\Delta B}$)-Fc is set forth in Figure 13A-13D (SEQ ID NOS: 3 and 4).

Please replace the paragraph starting on page 53, line 4, with the following:

A second deletion mutant construct, designated Mut2: Flt1(2-3$_{\Delta B}$)-Fc, was derived from the Mut1: Flt1(1-3$_{\Delta B}$)-Fc construct by deletion of Flt1 Ig domain 1 encoded by nucleotides 79-393 (see Figure 10A-10D [SEQ ID

NOS: 1 and 2]); for convenience, nucleotides 73-78 (TCA GGT) were changed to TCC GGA. This introduced a restriction site (BspE1) without altering the associated amino acid sequence, Ser-Gly. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), was also sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut2: Flt1(2-$3_{\Delta B}$)-Fc is set forth in Figure 14A-14C (SEQ ID NOS: 5 and 6).

Please replace the paragraph starting on page 53, line 23, through page 54, line 4, with the following:

A third deletion mutate construct, designated Mut3: Flt1(2-3)-Fc, was constructed the same way as the Mut2: Flt1(2-$3_{\Delta B}$)-Fc construct, except that Flt1 Ig domain 3 was left intact (the basic region amino acids were not deleted). The construct was constructed using standard molecular biology techniques and the final construct was sequence-verified as described *supra.* The sequence of Mut3: Flt1(2-3)-Fc is set forth in Figure 15A-15C (SEQ ID NOS: 7 and 8).

Please replace the paragraph starting on page 54, line 9, with the following:

A final construct was made in which a N-glycosylation site was introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1(1-3$_{R->N}$)-Fc and was made by changing nucleotides 824-825 from GA to AC, consequently changing the coded Arg residue (AGA) into an Asn residue (AAC) (see Figure 10A-10D [SEQ ID NOS: 1 and 2]). The resulting amino acid sequence is therefore changed from Arg-Ala-Ser to Asn-Ala-Ser, which matches the canonical signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue. The sequence of Mut4: Flt1(1-3$_{R->N}$)-Fc is set forth in Figure 16A-16D (SEQ ID NOS: 9 and 10).

Please replace the paragraph starting on page 60, line 4, with the following:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3' [SEQ ID NO: 18])

Please replace the paragraph starting on page 60, line 6, with the

3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAGCTGGATATCTATGATTGTA TTGGT [SEQ ID NO: 19])

Please replace the paragraph starting on page 60, line 8, with the following:

The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO: 20) (corresponding to amino acids 27-33 of Figure 21A-21C [SEQ ID NOS: 11 and 12]). The 3' primer encodes the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID (SEQ ID NO: 37)of Flt1 (corresponding to amino acids 123-126 of Figure 21A-21C [SEQ ID NOS: 11 and 12]) and continuing into VVLS (SEQ ID NO: 38) (corresponding to amino acids 127-130 of Figure 21A-21C [SEQ ID NOS: 11 and 12]) of Flk1.

Please replace the paragraph starting on page 60, line 20, with the following:

5': Flt1D2-Flk1D3.s (5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATG G-3' [SEQ ID NO: 21])

Please replace the paragraph starting on page 60, line 23, with the following:

3': Flk1D3/apa/srf.as (5'-GATAATGCCCGGGCCCTTTTCATGGACCCTGAC AAATG-3' [SEQ ID NO: 22])

Please replace the paragraph starting on page 61, line 1, with the following:

The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (SEQ ID NO: 23) (corresponding to amino acids 223-228 of Figure 21A-21C [SEQ ID NOS: 11 and 12]), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of Figure 21A-21C (SEQ ID NOS: 11 and 12).

Please replace the paragraph starting on page 61, line 10, with the following:

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described *supra*) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 614bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ΔB2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested

with the restriction enzymes EcoRI and SrfI and the resulting 702bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-FcΔC1(a) to produce the plasmid pFlt1D2.Flk1D3.FcΔC1(a). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.FcΔC1(a) chimeric molecule is set forth in Figure 21A-21C [SEQ ID NOS: 11 and 12].

Please replace the paragraph starting on page 62, line 13, with the following:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3' [SEQ ID NO: 24])

Please replace the paragraph starting on page 62, line 15, with the following:

3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAGCTGGATATCTATGATTGTA TTGGT [SEQ ID NO: 25])

Please replace the paragraph starting on page 62, line 18, with the following:

The 5' amplification primer encodes a BspE1 restriction site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO: 20) (corresponding to amino acids 27-33 of Figure 22A-22C [SEQ ID

NOS: 13 and 14]). The 3' amplification primer encodes the reverse complement of the end of Flt1 Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with the fusion point defined as TIID (SEQ ID NO: 37) of Flt1 (corresponding to amino acids 123-126 of Figure 22A-22C [SEQ ID NOS: 13 and 14]) and continuing into IQLL (SEQ ID NO: 26) of VEGFR3 (corresponding to amino acids 127-130 of Figure 22A-22C [SEQ ID NOS: 13 and 14]).

Please replace the paragraph starting on page 63, line 5, with the following:

5': R3D3.s (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA [SEQ ID NO: 27])

Please replace the paragraph starting on page 63, line 7, with the following:

3': R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG [SEQ ID NO: 28])

Please replace the paragraph starting on page 63, line 16, with the following:

5':Flt1D2.VEGFR3D3.s
(TCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAG [SEQ ID NO: 29])

Please replace the paragraph starting on page 63, line 19, with the following:

3': VEGFR3D3/srf.as
(GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT [SEQ ID NO: 30])

Please replace the paragraph starting on page 63, line 22, through page 64, line 4, with the following:

The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (SEQ ID NO: 31) (corresponding to amino acids 221-226 of Figure 22A-22C [SEQ ID NOS: 13 and 14]), followed by a bridging sequence that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of Figure 22A-22C (SEQ ID NOS: 13 and 14).

Please replace the paragraph starting on page 64, line 6, with the following:

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were

combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described *supra*, to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 625bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/Flt1ΔB2.Fc (described *supra*), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc. The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 693bp fragment was subcloned into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)ΔB2-FcΔC1(a) to produce the plasmid designated pFlt1D2.VEGFR3D3.FcΔC1(a). The complete DNA deduced amino acid sequence of the Flt1D2.VEGFR3D3.FcΔC1(a) chimeric molecule is set forth in Figure 22A-22C (SEQ ID NOS: 13 and 14).

Please replace the paragraph starting on page 67, line 7, with the following:

The pVEGFR1R2.FcΔC1(a) expression plasmid was constructed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of Figure 24A-24C [SEQ ID NOS: 15 and 16]) between Flt1d2-Flk1d3-FcΔC1(a) amino acids 26 and 27 of Figure 21A-21C (SEQ ID NOS: 11 and 12) (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231 of Figure 21A-21C (SEQ ID NOS: 11 and 12). The SDT amino acid sequence is native to the Flt1 receptor and

was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2.FcΔC1(a) chimeric molecule is set forth in Figure 24A-24C (SEQ ID NOS: 15 and 16).

Please replace the paragraph starting on page 80, line 22, through page 81, line 6, with the following:

The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. In this method, the protein was first cleaved with trypsin. Tryptic fragments were analyzed and identified by HPLC coupled with mass spectrometry, in addition to an N-terminal sequencing technique. Reduction of the tryptic digest was employed to help identify disulfide-bond-containing fragments. Treatment of the tryptic digest with PNGase F (Glyko, Novato, CA) was employed to help identify fragments with N-linked glycosylation sites. The results are summarized in the accompanying Figure 36 (SEQ ID NO: 17).

Please replace the paragraph starting on page 81, line 20, through page 82, line 2, with the following:

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino

acid sequence NIT), Asn193 (amino acid sequence NST), and Asn282 (amino acid sequence NST). In addition, partial glycosylation isobserved on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the Figure 36 (SEQ ID NO: 17).

Please replace the paragraph starting on page 89, line 13, with the following:

10pM of VEGF165 was incubated overnight at room temperature with modified Flt1 receptor variants ranging from 160pM to 0.1pM. The modified Flt1 receptor variants used in this experiment were Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), transiently expressed Flt1D2Flk1D3.FcΔC1(a), transiently expressed Flt1D2VEFGFR3D3-FcΔC1(a), Flt1-(1-3_{NAS})-Fc, Flt1(1-$3_{R->C}$)-Fc and Tie2-Fc. Flt1(1-3_{NAS})-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic amino acid sequence KNKRASVRRR (SEQ ID NO: 32) is replaced by NASVNGSR (SEQ ID NO: 33), resulting in the incorporation of two new glycosylation sites and a net reduction of five positive charges, both with the purpose of reducing the unfavorable effects of this sequence on PK. Flt1(1-$3_{R->C}$)-Fc is a modification in which a single arginine (R) residue within the same basic amino acid sequence is changed to a cysteine (C) (KNKRASVRRR (SEQ ID NO: 32) -> KNKCASVRRR [SEQ ID NO: 34]) to allow for pegylation at that residue, which could then shield the basic region from exerting its unfavorable effects on PK. After incubation the solution was transferred to a plate containing a capture antibody for VEGF165 (R&D). The amount of free

VEGF165 was then determined using an antibody to report free VEGF165. This showed that the modified Flt1 receptor variant with the highest affinity for VEGF165 (determined as the lowest amount of free VEGF165) was Flt1D2Flk1D3.FcΔC1(a), followed by Flt1(1-3)-Fc and Flt1(1-3)-Fc (A40) and then by Flt1(1-$3_{R->C}$)-Fc, Flt1(1-3_{NAS})-Fc and Flt1D2VEFGFR3D3-FcΔC1(a). Tie2Fc has no affinity for VEGF165.

REMARKS

This Preliminary Amendment is made merely to insert the priority data and to add the sequence identifiers to the specification.
Applicants submit herewith as Exhibit A: Marked-Up Versions of pages 1, 11, 13, 15, 19, 20, 21, 22, 25, 49, 50, 51, 52, 53, 54, 60, 61, 62, 63, 64, 67, 80, 81, 82, 89, 90, 92, 93, 95, 98, and 99; Exhibit B: Sequence Listings in paper and computer readable forms.

I hereby state that the content of the paper readable and computer readable copy of the Sequence Listing submitted herewith and referred to herein in accordance with 37 C.F.R. § 1.821(g), contain no new subject matter.

Applicants direct the subject Sequence Listings submitted herewith be added to the specification.

Att. Docket No.: REG 710-A-US
USSN: Not Yet Known
US File Date: Filed Herewith
Preliminary Amendment

No fee is deemed necessary for filing this paper. However, if any fees are deemed necessary, the Commissioner is hereby authorized to charge any such fees required by this paper to Deposit Account No. 18-0650.

Respectfully submitted,

Gail M. Kempler
Reg. No. 32,143
Joseph M. Sorrentino
Reg. No. 32,598
Attorneys for Applicants
Linda O. Palladino
Reg. No. 45,636
Patent Agent for Applicants
Regeneron Pharmaceuticals, Inc.
777 Old Saw Mill River Road
Tarrytown, New York 10591
(914) 345-7400

MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES AND METHODS OF MAKING AND USING THEREOF

This application claims priority of International Application No. PCT/US00/14142 filed, May 23, 2000, which ~~The application~~ claims priority of U.S. Provisional Application No. 60/138,133, filed on June 8, 1999. Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

INTRODUCTION

The field of this invention is modified polypeptides with improved pharmacokinetics. Specifically, the field of this invention relates to Flt1 receptor polypeptides that have been modified in such a way as to improve their pharmacokinetic profile. The field of this invention also relates to methods of making and using the modified polypeptides including but not limited to using the modified polypeptides to decrease or inhibit plasma leakage and/or vascular permeability in a mammal.

BACKGROUND

The ability of polypeptide ligands to bind to cells and thereby elicit a phenotypic response such as cell growth, survival, cell product secretion, or differentiation is often mediated through transmembrane receptors on the cells. The extracellular domain of such receptors (i.e. that portion of the receptor that is displayed on the surface of the cell) is generally the most distinctive portion of the molecule, as it provides the protein with its ligand binding characteristic. Binding of a ligand

In still another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

In a preferred embodiment of the invention, the multimerizing component comprises an immunoglobulin domain.

In another embodiment, the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include an isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of

(a) the nucleotide sequence set forth in Figure 13A-13D; (SEQ ID NOS: 3 and 4)
(b) the nucleotide sequence set forth in Figure 14A-14C; (SEQ ID NOS: 5 and 6)
(c) the nucleotide sequence set forth in Figure 15A-15C; (SEQ ID NOS: 7 and 8)
(d) the nucleotide sequence set forth in Figure 16A-16D; (SEQ ID NOS: 9 and 10)
(e) the nucleotide sequence set forth in Figure 21A-21C; (SEQ ID NOS: 11 and 12)
(f) the nucleotide sequence set forth in Figure 22A-22C; (SEQ ID NOS: 13 and 14)
(g) the nucleotide sequence set forth in Figure 24A-24C; and (SEQ ID NOS: 15 and 16)
(h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c), (d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having

Another embodiment of the invention is a method of producing a fusion polypeptide which comprises growing cells of the host-vector system under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

Additional embodiments include a fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D (SEQ ID NOS: 1 and 2) or Figure 24A-24C, (SEQ ID NOS: 15 AND 16 which has been modified by acetylation or pegylation wherein the acetylation is accomplished with at least about a 100 fold molar excess of acetylation reagent or wherein acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess or wherein the pegylation is 10K or 20K PEG.

A preferred embodiment includes a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above, including embodiments wherein the mammal is a human, the fusion polypeptide is acetylated or the fusion polypeptide is pegylated.

A further embodiments is a fusion polypeptide which specifically binds the VEGF receptor ligand VEGF.

A preferred embodiment of the invention is a method of blocking blood vessel growth in a human comprising administering an effective amount of the fusion polypeptide described above.

Still another embodiment of the fusion polypeptide is one in which the second VEGF receptor is Flt4.

Preferred embodiments include a fusion polypeptide wherein amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor and a fusion polypeptide wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

In yet another embodiment, the fusion polypeptide multimerizing component comprises an immunoglobulin domain including an embodiment wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include a fusion polypeptide comprising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from the group consisting of (a) the amino acid sequence set forth in Figure 13A-13D; (SEQ ID NOS: 3 AND 4) (b) the amino acid sequence set forth in Figure 14A-14C; (SEQ ID NOS: 5 AND 6) (c) the amino acid sequence set forth in Figure 15A-15C; (SEQ ID NOS: 7 and 8) (d) the amino acid sequence set forth in Figure 16A-16D; (SEQ ID NOS: 9 AND 10) (e) the amino acid sequence set forth in Figure 21A-21C; (SEQ ID NOS: 11 AND 12) (f) the amino acid sequence set forth in Figure 22A-22C; (SEQ ID NOS: 13 AND 14) and (g) the amino acid sequence set forth in Figure 24A-24C. (SEQ ID NOS: 15 AND 16)

unmodified, 10, 20 and 40 fold excess samples and 2 mice for 60 and 100 fold excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1(1-3)-Fc. The T_{max} for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the C_{max} was as follows: Unmodified Flt1(1-3)-Fc: 0.06μg/ml; 10 fold excess sample: - 0.7μg/ml, 20 fold excess sample - 2μg/ml, 40 fold excess sample - 4μg/ml, 60 fold excess sample - 2μg/ml, 100 fold excess sample - 1μg/ml.

(SEQ ID NOS: 1 and 2) → (SEQ ID NO: 1) (SEQ ID NO: 2

Figure 10A-10D. Nucleic acid and deduced amino acid sequence of Flt1(1-3)-Fc.

Figure 11. Schematic diagram of the structure of Flt1.

Figure 12A and 12B. Hydrophilicity analysis of the amino acid sequences of Ig domain 2 and Ig domain 3 of Flt1.

(SEQ ID NOS: 3 and 4) (SEQ ID NO: 3) (SEQ ID NO: 4)

Figure 13A-13D. Nucleic acid and deduced amino acid sequence of Mut1: Flt1(1-$3_{\Delta B}$)-Fc.

(SEQ ID NOS: 5 and 6) (SEQ ID NO: 5) (SEQ ID NO: 6)

Figure 14A-14 C. Nucleic acid and deduced amino acid sequence of Mut2: Flt1(2-$3_{\Delta B}$)-Fc.

(SEQ ID NOS: 7 and 8) (SEQ ID NO: 7) (SEQ ID NO: 8)

Figure 15A-15C. Nucleic acid and deduced amino acid sequence of Mut3: Flt1(2-3)-Fc.

(SEQ ID NOS: 9 AND 10) (SEQ ID NO: 9) (SEQ ID NO: 10)

Figure 16A-16D. Nucleic acid and deduced amino acid sequence of Mut4: Flt1(1-3$_{R->N}$)-Fc.

Figure 17. Binding of unmodified Flt1(1-3)-Fc, basic region deletion mutant Flt1(1-3)-Fc, and Flt1(1-3)$_{R->N}$ mutant proteins in a Biacore-based assay. At the sub-stoichiometric ratio (0.25 μg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. μg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF to the Flt1(1-3)-Fc immobilized on the Biacore chip. At 0.5 μg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the Biacore chip. At 1.0 μg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, which is approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the Biacore chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-3$_{\Delta B}$)-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1(1-3$_{R->N}$)-Fc is somewhat less efficient at blocking binding

Figure 18. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, Mut2: Flt1(2-3$_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins to Matrigel® coated plates. Unmodified Flt1(1-3)-Fc protein binds avidly to these wells, the Mut3: Flt1(2-3)-Fc protein binds somewhat more weakly, the Mut1: Flt1(1-3$_{\Delta B}$)-Fc protein binds more weakly still, and the Mut2: Flt1(2-3$_{\Delta B}$)-Fc protein shows the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1(1-3$_{R->N}$)-Fc

glycosylation mutant protein shows only marginal benefit on the Matrigel assay.

Figure 19. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-$3_{\Delta B}$)-Fc, Mut2: Flt1(2-$3_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins in an ELISA-based assay. At the concentrations tested, unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-$3_{\Delta B}$)-Fc, Mut2: Flt1(2-$3_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins bind VEGF similarly.

Figure 20. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-$3_{\Delta B}$)-Fc, Mut2: Flt1(2-$3_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins. the Cmax for these reagents was as follows: Unmodified Flt1(1-3)-Fc - 0.15μg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc - 1.5μg/ml; and Mut1: Flt1(1-$3_{\Delta B}$)-Fc - 0.7μg/ml.

(SEQ ID NOS: 11 and 12) (SEQ ID NO: 11) (SEQ ID NO: 12)

Figure 21A-21C. Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.Flk1D3.FcΔC1(a).

(SEQ ID NOS: 13 and 14) (SEQ ID NO: 13) (SEQ ID NO: 14)

Figure 22A-22C. Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.VEGFR3D3.FcΔC1(a).

Figure 23. Extracellular Matrix (ECM) Assay. The results of this assay demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

(SEQ ID NOS: 15 and 16) (SEQ ID NO: 15) (SEQ ID NO: 16)

Figure 24A-24C. Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed VEGFR1R2-FcΔC1(a).

Figure 25A-25C. Phosphorylation assay. At a 1.5 molar excess of either Flt1(1-3)-Fc , Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.FcΔC1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.FcΔC1(a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similar results are seen in Figure 25B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In Figure 25C, where the modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.FcΔC1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

Figure 26A-26B. Phosphorylation assay. Detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are not phosphorylated by challenge samples which have VEGF165 preincubated with 1 and 2 fold molar excess (Figure 26A) or 3 and 4 fold molar excess (Figure 26B) of either transient Flt1D2Flk1D3.FcΔC1(a), stable Flt1D2Flk1D3.FcΔC1(a), or transient VEGFR1R2-FcΔC1(a). At all modified Flt1 receptor concentrations tested there is complete binding of VEGF165 ligand

MARKED-UP VERSION

Figure 33, Figure 34 and Figure 35. Size Exclusion Chromatography (SEC) with On-Line Light Scattering. Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa Barbara, California) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. As shown in Figure 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300 (Figure 33), the MW of VEGF165 at the peak position is 44 390 (Figure 34) and the MW of R1R2 at the peak is 113 300 (Figure 35).

(SEQ ID NO:17)

Figure 36. Peptide mapping and glycosylation analysis. The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 is disulfide bonded to Cys76. Cys121 is disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, it can not be determined

(d.) Pharmacokinetic analysis of step-acetylated Flt1(1-3)-Fc

In vivo experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc protein. Balb/c mice (23-28g) were injected subcutaneously with 4mg/kg of unmodified or 10, 20, 40, 60 and 100 fold molar excess samples of step-acetylated Flt1(1-3)-Fc (3 mice for unmodified, 10, 20 and 40 fold molar excess samples and 2 mice for 60 and 100 fold molar excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1(1-3)-Fc (described *supra*). Figure 9 details the results of this study. The Tmax for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the Cmax was as follows: Unmodified Flt1(1-3)-Fc: 0.06μg/ml; 10 fold molar excess sample: - 0.7μg/ml, 20 fold molar excess sample - 2μg/ml, 40 fold molar excess sample - 4μg/ml, 60 fold molar excess sample - 2μg/ml, 100 fold molar excess sample - 1μg/ml. This results demonstrates that acetylation or pegylation of Flt1(1-3)-Fc significantly improves its pharmacokinetic profile.

Example 11: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut1: Flt1(1-3$_{\Delta B}$)-Fc.

Based on the observation that acetylated Flt1(1-3)-Fc, which has a pI

below 6, has much better pharmacokinetics than the highly positive unmodified Flt1(1-3)-Fc (pI > 9.3), it was asked whether the difference in pharmacokinetics could be attributed to the net charge of the protein, which made it stick to negatively charged extracellular matrix components, or whether there were perhaps specific locations on the surface of the Flt1(1-3)-Fc protein that constituted specific binding sites for extracellular matrix components. For example, many proteins are known to have heparin binding sites, often consisting of a cluster of basic residues. Sometimes these residues are found in a cluster on the primary sequence of the protein; some of the literature has identified "consensus sequences" for such heparin binding sites (see for example Hileman, et al., 1998, Bioessays 20(2):156-67). In other cases, the known crystal structure of a protein reveals a cluster of positively charged residues on the surface of a protein, but the residues come from different regions of the primary sequence and are only brought together when the protein folds into its tertiary structure. Thus it is difficult to deduce whether an isolated amino acid residue forms part of a cluster of basic residues on the surface of the protein. However, if there is a cluster of positively charged amino acid residues in the primary sequence, it is not unreasonable to surmise that the residues are spatially close to one another and might therefore be part of an extracellular matrix component binding site. Flt1 receptor has been studied extensively and various domains have been described (see for example Tanaka et al., 1997, Jpn. J. Cancer Res 88:867-876). Referring to the nucleic acid and amino acid sequence set forth in Figure 10A-10D (SEQ ID NOS: 1 and 2)

of this application, one can identify the signal sequence for secretion which is located at the beginning of the sequence and extends to the glycine coded for by nucleotides 76-78. The mature protein begins with Ser-Lys-Leu-Lys (SEQ ID NO. 35), starting at nucleotide 79 of the nucleic acid sequence. Flt1 Ig domain 1 extends from nucleotide 79 to 393, ending with the amino acids Ser-Asp-Thr. Flt1 Ig domain 2 extends from nucleotide 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1 Ig domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val to Asp-Lys-Ala). There is a bridging amino acid sequence, Gly-Pro-Gly, encoded by nucleotides 997-1005, followed by the nucleotide sequence encoding human Fc (nucleotides 1006-1701 or amino acids Glu-Pro-Lys to Pro-Gly-Lys-stop).

A more detailed analysis of the Flt1 amino acid sequence reveals that there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR) [SEQ ID NO 36] of Figure 10A-10D (SEQ ID NOS: 1 and 2), in which 6 out of 10 amino acid residues are basic. This sequence is located in Flt1 Ig domain 3 of the receptor (see Figure 11), which is not itself essential for binding of VEGF ligand, but which confers a higher affinity binding to ligand. An alignment of the sequence of Ig domain 3 with that of Ig domain 2 reveals that in this region, there is very poor alignment between the two Ig domains, and that there are about 10 additional amino acids in Ig domain 3. An analysis of the hydrophilicity profiles (MacVector computer software) of these two domains clearly indicates the presence of a hydrophilic region in the protein (Figure 12A-12B). These observations raised the

possibility that the actual three dimensional conformation of Flt1 Ig domain 3 allowed for some type of protrusion that is not in Flt1 Ig domain 2. To test this hypothesis, the 10 additional amino acids were deleted and the resulting protein was tested to see whether the deletion would affect the pharmacokinetics favorably without seriously compromising the affinity of the receptor for VEGF. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), is referred to as Mut1: Flt1(1-$3_{\Delta B}$)-Fc. The Mut1: Flt1(1-$3_{\Delta B}$)-Fc construct was derived from Flt1(1-3)-Fc by deletion of nucleotides 814-843 (set forth in Figure 10A-10D) (SEQ ID NOS. 1 AND 2), which deletes the highly basic 10-amino acid residue sequence Lys-Asn-Lys-Arg-Ala-Ser-Val-Arg-Arg-Arg (SEQ ID NO. 32) from Flt1 Ig domain 3.

The final DNA construct was sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut1: Flt1(1-$3_{\Delta B}$)-Fc is set forth in Figure 13A-13D (SEQ ID NOS. 3 AND 4).

Example 12: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut2: Flt1(2-3 $_{\Delta B}$)-Fc.

A second deletion mutant construct, designated Mut2: Flt1(2-3$_{\Delta B}$)-Fc, was derived from the Mut1: Flt1(1-3$_{\Delta B}$)-Fc construct by deletion of Flt1 Ig domain 1 encoded by nucleotides 79-393 (see Figure 10A-10D (SEQ ID NOS: 1 and 2)); for convenience, nucleotides 73-78 (TCA GGT) were changed to TCC GGA. This introduced a restriction site (BspE1) without altering the associated amino acid sequence, Ser-Gly. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), was also sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut2: Flt1(2-3$_{\Delta B}$)-Fc is set forth in Figure 14A-14C (SEQ ID NOS: 5 and 6).

Example 13: Construction of Flt1(1-3)-Fc deletion mutant designated Mut3: Flt1(2-3)-Fc.

A third deletion mutate construct, designated Mut3: Flt1(2-3)-Fc, was constructed the same way as the Mut2: Flt1(2-3$_{\Delta B}$)-Fc construct, except that Flt1 Ig domain 3 was left intact (the basic region amino

acids were not deleted). The construct was constructed using standard molecular biology techniques and the final construct was sequence-verified as described *supra*. The sequence of Mut3: Flt1(2-3)-Fc is set forth in Figure 15A-15C. (SEQ ID NOS: 7 and 8)

Example 14: Construction of Flt(1-3)-Fc basic region N-glycosylation mutant designated Mut4: Flt1(1-3 $_{R->N}$)-Fc.

A final construct was made in which a N-glycosylation site was introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1(1-3$_{R->N}$)-Fc and was made by changing nucleotides 824-825 from GA to AC, consequently changing the coded Arg residue (AGA) into an Asn residue (AAC) (see Figure 10A-10D). [SEQ ID NOS 1 and 2] The resulting amino acid sequence is therefore changed from Arg-Ala-Ser to Asn-Ala-Ser, which matches the canonical signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue. The sequence of Mut4: Flt1(1-3$_{R->N}$)-Fc is set forth in Figure 16A-16D. (SEQ ID NOS: 9 and 10)

Example 15: Characterization of acetylated Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, and Mut4: Flt1(1-3$_{R->N}$)-Fc mutants.

(a.) Binding to extracellular matrix components

To determine whether the three modified proteins were more or less

single fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

[SEQ ID NO: 18]

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')

[SEQ ID NO: 19]

3': Flt1D2-Flk1D3.as (5'-CGGACTCAGAACCACATCTATGATTGTATTGGT-3')

The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence (SEQ ID NO: 20) GRPFVEM (corresponding to amino acids 27-33 of Figure 21A-21C [SEQ ID NOS: 11 and 12]). The 3' primer encodes the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID (SEQ ID NO: 37) of Flt1 (corresponding to amino acids 123-126 of Figure 21A-21C [SEQ ID NOS: 11 and 12]) and continuing into VVLS (SEQ ID NO: 38) (corresponding to amino acids 127-130 of Figure 21A-21C [SEQ ID NOS. 11 and 12]) of Flk1.

For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as follows:

5': Flt1D2-Flk1D3.s (5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATG G-3') [SEQ ID NO: 21]

3': Flk1D3/apa/srf.as (5'-GATAATGCCCGGGCCCTTTTCATGGACCCTGAC AAATG-3') [SEQ ID NO: 22]

The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (SEQ ID NO. 23) (corresponding to amino acids 223-228 of Figure 21A-21C [SEQ ID NOS: 11 and 12]), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of Figure 21A-21C. (SEQ ID NOS: 11 and 12)

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described *supra*) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 614bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ΔB2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 702bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-FcΔC1(a) to produce the plasmid pFlt1D2.Flk1D3.FcΔC1(a). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.FcΔC1(a) chimeric molecule is set forth in Figure 21A-21C. (SEQ ID NOS: 11 and 12)

(b) Construction of the expression plasmid pFlt1D2VEGFR3D3FcΔC1(a)

The expression plasmid pMT21.Flt1(1-3).Fc (6519bp) encodes ampicillin resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1 receptor. This plasmid was used to produce a DNA fragment containing Ig domain 2 of Flt1 by PCR. RNA from the cell line HEL921.7 was used to produce Ig domain 3 of Flk1, using standard RT-PCR methodology. A further round of PCR amplification was used to achieve fusion of the two Ig domains into a single fused fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3' [SEQ ID NO: 24])

3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAGCTGGATATCTATGATTGTA TTGGT [SEQ ID NO: 25])

The 5' amplification primer encodes a BspE1 restriction site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (SEQ ID NO: 20) (corresponding to amino acids 27-33 of Figure 22A-22C [SEQ ID NOS: 13 AND 14]). The 3' amplification primer encodes the reverse complement of the end of Flt1 Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with the fusion point defined as TIID (SEQ ID NO: 37) of Flt1 (corresponding to amino acids 123-126 of Figure 22A-22C [SEQ ID NOS: 13 AND 14]) and continuing into IQLL (SEQ ID NO: 26) of VEGFR3 (corresponding to amino acids 127-130 of Figure 22A-22C [SEQ ID NOS: 13 AND 14]).

MARKED-UP VERSION

For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as follows:

[SEQ ID NO:27]

5': R3D3.s (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA)

[SEQ ID NO: 28]

3': R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG)

Both the 5' and 3' amplification primers match the sequence of VEGFR3. The 296bp amplification product of this RT-PCR reaction was isolated by standard techniques and subjected to a second round of PCR to add suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The amplification primers were as follows:

5':Flt1D2.VEGFR3D3.s [SEQ ID NO: 29]

(TCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAG)

3': VEGFR3D3/srf.as [SEQ ID NO: 30]

(GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT)

The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of (SEQ ID NO: 31) VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (corresponding

[SEQ ID NOS: 13 and 14]

to amino acids 221-226 of Figure 22A-22C), followed by a bridging sequence that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of Figure 22A-22C. (SEQ ID NOS: 13 and 14)

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described *supra*, to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 625bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/Flt1ΔB2.Fc (described *supra*), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc. The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 693bp fragment was subcloned into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)ΔB2-FcΔC1(a) to produce the plasmid designated pFlt1D2.VEGFR3D3.FcΔC1(a). The complete DNA deduced amino acid sequence of the Flt1D2.VEGFR3D3.FcΔC1(a) chimeric molecule is set forth in Figure 22A-22C. (SEQ ID NOS: 13 and 14)

cells. The supernatant was decanted into sterile 1L bottles and purification of the expressed protein was performed as described *infra.*

Example 20: Construction pVEGFR1R2-FcΔC1(a) expression vector

The pVEGFR1R2.FcΔC1(a) expression plasmid was constructed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of Figure 24A-24C [SEQ ID NOS: 15 and 16]) between Flt1d2-Flk1d3-FcΔC1(a) amino acids 26 and 27 of Figure 21A-21C (SEQ ID NOS: 11 and 12) (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231 of Figure 21A-21C (SEQ ID NOS: 11 and 12). The SDT amino acid sequence is native to the Flt1 receptor and was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2.FcΔC1(a) chimeric molecule is set forth in Figure 24A-24C (SEQ ID NOS: 15 and 16).

Example 21: Cell Culture Process Used to Produce Modified Flt1 Receptors

(a) Cell Culture Process Used to Produce Flt1D2.Flk1D3.FcΔC1(a)

The process for production of Flt1D2.Flk1D3.FcΔC1(a) protein using the

(Pharmacia) equilibrated in PBS buffer and eluted with the same buffer at flow rate 0.5 ml/min. at room temperature. As shown in Figure 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300 (Figure 33), the MW of VEGF165 at the peak position is 44 390 (Figure 34) and the MW of R1R2 at the peak is 113 300 (Figure 35).

These data indicated that the stoichiometry of the Flt1D2Flk1D3.FcΔC1(a)/VEGF complex is 1:1 as its corresponds to the sum of molecular weights for Flt1D2Flk1D3.FcΔC1(a) and VEGF165. Importantly, this method conclusively proved that the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex was indeed composed of only one molecule of VEGF165 ligand and only one molecule of the Flt1D2Flk1D3.FcΔC1(a).

Example 28: Peptide Mapping of Flt1D2.Flk1D3.FcΔC1(a)

The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. In this method, the protein was first cleaved with trypsin. Tryptic fragments were analyzed and identified by HPLC coupled with mass

spectrometry, in addition to an N-terminal sequencing technique. Reduction of the tryptic digest was employed to help identify disulfide-bond-containing fragments. Treatment of the tryptic digest with PNGase F (Glyko, Novato, CA) was employed to help identify fragments with N-linked glycosylation sites. The results are summarized in the accompanying Figure 36. (SEQ ID NO: 17)

There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 has been confirmed to be disulfide bonded to Cys76. Cys121 is confirmed to be disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, because these two cysteines can not be separated enzymatically from each other, it can not be determined whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is confirmed to be disulfide bonded to Cys306. Cys 352 is confirmed to be disulfide bonded to Cys410.

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino acid sequence NIT), Asn193 (amino acid sequence NST), and Asn282 (amino acid sequence NST). In addition, partial glycosylation is

observed on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the Figure 36. (SEQ ID NO: 17)

Example 29: Pharmacokinetic Analysis of Modified Flt Receptors

(a) Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)

Balb/c mice (25-30g) were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a), CHO stably expressed Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently expressed VEGFR1R2-FcΔC1(a). The mice were tail bled at 1, 2, 4, 6, 24hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a). The ELISA involves coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. The results of this experiments are shown in Figure 37. The T_{max} for Flt1(1-3)-Fc (A40) was at 6 hrs while the T_{max} for the transient and stable Flt1D2.Flk1D3.FcΔC1(a) and the transient VEGFR1R2-FcΔC1(a) was 24hrs. The C_{max} for Flt1(1-3)-Fc (A40) was 8μg/ml. For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the C_{max}

Example 33: Pharmacokinetic Analysis of Flt1(1-3)-Fc (A40) and Pegylated Flt1(1-3)-Fc

Flt1(1-3)-Fc was PEGylated with either 10kD PEG or 20kD PEG and tested in balb/c mice for their pharmacokinetic profile. Both PEGylated forms of Flt1(1-3)-Fc were found to have much better PK profiles than Flt1(1-3)-Fc (A40), with the Tmax occurring at 24 hrs. for the PEGylated molecules as opposed to 6 hrs. for Flt1(1-3)-Fc (A40).

Example 34: VEGF165 ELISA to Test Affinity of Modified Flt1 Receptor Variants

10pM of VEGF165 was incubated overnight at room temperature with modified Flt1 receptor variants ranging from 160pM to 0.1pM. The modified Flt1 receptor variants used in this experiment were Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), transiently expressed Flt1D2Flk1D3.FcΔC1(a), transiently expressed Flt1D2VEFGFR3D3-FcΔC1(a), Flt1-(1-3_{NAS})-Fc, Flt1(1-$3_{R->C}$)-Fc and Tie2-Fc. Flt1(1-3_{NAS})-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic amino acid sequence KNKRASVRRR (SEQ ID NO:32) is replaced by NASVNGSR (SEQ ID NO. 33), resulting in the incorporation of two new glycosylation sites and a net reduction of five positive charges, both with the purpose of reducing the unfavorable effects of this sequence on PK. Flt1(1-$3_{R->C}$)-Fc is a modification in which a single arginine (R) residue within the same basic amino acid sequence is changed to a cysteine (C) (KNKRASVRRR -> (SEQ ID NO. 34)

[SEQ ID NO: 347]

KNKCASVRRR) to allow for pegylation at that residue, which could then shield the basic region from exerting its unfavorable effects on PK. After incubation the solution was transferred to a plate containing a capture antibody for VEGF165 (R&D). The amount of free VEGF165 was then determined using an antibody to report free VEGF165. This showed that the modified Flt1 receptor variant with the highest affinity for VEGF165 (determined as the lowest amount of free VEGF165) was Flt1D2Flk1D3.FcΔC1(a), followed by Flt1(1-3)-Fc and Flt1(1-3)-Fc (A40) and then by Flt1(1-$3_{R->C}$)-Fc, Flt1(1-3_{NAS})-Fc and Flt1D2VEFGFR3D3-FcΔC1(a). Tie2Fc has no affinity for VEGF165.

5. The isolated nucleic acid molecule of claim 1, wherein the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

6. The isolated nucleic acid molecule of claim 1, wherein the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

7. The isolated nucleic acid molecule of claim 1, wherein the multimerizing component comprises an immunoglobulin domain.

8. The isolated nucleic acid molecule of claim 1, wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

9. (AMENDED) An isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in Figure 13A-13D; (SEQ ID NOS: 3 and 4)

(b) the nucleotide sequence set forth in Figure 14A-14C; (SEQ ID NOS: 5 and 6

(c) the nucleotide sequence set forth in Figure 15A-15C; (SEQ ID NOS: 7 AND 8)

(d) the nucleotide sequence set forth in Figure 16A-16D; (SEQ ID NOS: 9 AND 10)

(e) the nucleotide sequence set forth in Figure 21A-21C; (SEQ ID NOS: 11 and 12);

(f) the nucleotide sequence set forth in Figure 22A-22C; (SEQ ID NOS: 13 AND 14)

(g) the nucleotide sequence set forth in Figure 24A-24C; (SEQ ID NOS: 15 AND 16) and

(h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c), (d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having the biological activity of the modified Flt1 receptor fusion polypeptide.

10. A fusion polypeptide encoded by the isolated nucleic acid molecule of claim 1, 2, 3, 4 or 9.

11. A composition capable of binding a VEGF molecule to form a nonfunctional complex comprising a multimer of the fusion polypeptide of claim 10.

12. The composition of claim 11, wherein the multimer is a dimer.

13. The composition of claim 12 and a carrier.

14. A vector which comprises the nucleic acid molecule of claim 1, 2, 3, 4 or 9.

(AMENDED) 22. A fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D (SEQ ID NOS: 1 and 2) or Figure 24A-24C (SEQ ID NOS: 15 AND 16), which has been modified by acetylation or pegylation.

23. The fusion polypeptide of claim 22 wherein the modification is acetylation.

24. The fusion polypeptide of claim 22 wherein the modification is pegylation.

25. The fusion polypeptide of claim 23 wherein the acetylation is accomplished with at least about a 100 fold molar excess of acetylation reagent.

26. The fusion polypeptide of claim 23 wherein acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess.

27. The fusion polypeptide of claim 24 wherein the pegylation is 10K or 20K PEG.

28. A method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal fusion polypeptide of claim 10.

43. The fusion polypeptide of claim 41 wherein the second VEGF receptor is Flk1.

44. The fusion polypeptide of claim 41 wherein the second VEGF receptor is Flt4.

45. The fusion polypeptide claim 41, wherein amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

46. The fusion polypeptide of claim 41, wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

47. The fusion polypeptide of claim 41, wherein the multimerizing component comprises an immunoglobulin domain.

48. The fusion polypeptide of claim 41, wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

(AMENDED)

49. An fusion polypeptide comprising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from

the group consisting of:

(a) the amino acid sequence set forth in Figure 13A-13D; (SEQ ID NOS: 3 AND 4)

(b) the amino acid sequence set forth in Figure 14A-14C; (SEQ ID NOS: 5 AND 6)

(c) the amino acid sequence set forth in Figure 15A-15C; (SEQ ID NOS: 7 and 8)

(d) the amino acid sequence set forth in Figure 16A-16D; (SEQ ID NOS: 9 AND 10)

(e) the amino acid sequence set forth in Figure 21A-21C; (SEQ ID NOS: 11 and 12);

(f) the amino acid sequence set forth in Figure 22A-22C; and (SEQ ID NOS: 13 AND 14)

(g) the amino acid sequence set forth in Figure 24A-24C. (SEQ ID NOS: 15 AND 16)

50. A method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal fusion polypeptide of claim 41, 42, 43, 44 or 49.

51. A method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide of claim 41, 42, 43, 44 or 49.

MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES

The application claims priority of U.S. Provisional Application No. 60/138,133, filed on June 8, 1999. Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

INTRODUCTION

The field of this invention is modified polypeptides with improved pharmacokinetics. Specifically, the field of this invention relates to Flt1 receptor polypeptides that have been modified in such a way as to improve their pharmacokinetic profile. The field of this invention also relates to methods of making and using the modified polypeptides including but not limited to using the modified polypeptides to decrease or inhibit plasma leakage and/or vascular permeability in a mammal.

BACKGROUND

The ability of polypeptide ligands to bind to cells and thereby elicit a phenotypic response such as cell growth, survival, cell product secretion, or differentiation is often mediated through transmembrane receptors on the cells. The extracellular domain of such receptors (i.e. that portion of the receptor that is displayed on the surface of the cell) is generally the most distinctive portion of the molecule, as it provides the protein with its ligand binding characteristic. Binding of a ligand

to the extracellular domain generally results in signal transduction which transmits a biological signal to intracellular targets. Often, this signal transduction acts via a catalytic intracellular domain. The particular array of sequence motifs of this catalytic intracellular domain determines its access to potential kinase substrates (Mohammadi, et al.,1990, Mol. Cell. Biol. 11:5068-5078; Fantl, et al., 1992, Cell 69:413-413). Examples of receptors that transduce signals via catalytic intracellular domains include the receptor tyrosine kinases (RTKs) such as the Trk family of receptors which are generally limited to cells of the nervous system, the cytokine family of receptors including the tripartate CNTF receptor complex (Stahl & Yancopoulos, 1994, J. Neurobio. 25:1454-1466) which is also generally limited to the cells of the nervous system, G-protein coupled receptors such as the β_2-adrenergic receptor found on, for instance, cardiac muscle cells, and the multimeric IgE high affinity receptor FcεRI which is localized, for the most part, on mast cells and basophils (Sutton & Gould, 1993, Nature 366:421-428).

All receptors identified so far appear to undergo dimerization, multimerization, or some related conformational change following ligand binding (Schlessinger, J., 1988, Trend Biochem. Sci. 13:443-447; Ullrich & Schlessinger, 1990, Cell 61:203-212; Schlessinger & Ullrich, 1992, Neuron 9:383-391) and molecular interactions between dimerizing intracellular domains lead to activation of catalytic function. In some instances, such as platelet-derived growth factor (PDGF), the ligand is a dimer that binds two receptor molecules (Hart, et al., 1988, Science, 240:1529-1531; Heldin, 1989, J. Biol. Chem. 264:8905-8912) while, for example, in the case of epidermal growth

factor (EGF), the ligand is a monomer (Weber, et al., 1984, J. Biol. Chem. 259:14631-14636). In the case of the FcεRI receptor, the ligand, IgE, exists bound to FcεRI in a monomeric fashion and only becomes activated when antigen binds to the IgE/FcεRI complex and cross-links adjacent IgE molecules (Sutton & Gould, 1993, Nature 366:421-428).

Often, the tissue distribution of a particular receptor within higher organisms provides insight into the biological function of the receptor. The RTKs for some growth and differentiation factors, such as fibroblast growth factor (FGF), are widely expressed and therefore appear to play some general role in tissue growth and maintenance. Members of the Trk RTK family (Glass & Yancopoulos, 1993, Trends in Cell Biol. 3:262-268) of receptors are more generally limited to cells of the nervous system, and the Nerve Growth Factor family consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), which bind the Trk RTK family receptors, promote the differentiation of diverse groups of neurons in the brain and periphery (Lindsay, R. M, 1993, in Neurotrophic Factors, S.E. Loughlin & J.H. Fallon, eds., pp. 257-284, San Diego, CA, Academic Press). FcεRI is localized to a very limited number of types of cells such as mast cells and basophils. Mast cells derive from bone marrow pluripotent hematopoietic stem cell lineage, but complete their maturation in the tissue following migration from the blood stream (See Janeway & Travers, 1996, in Immunobiology, 2d. Edition, M. Robertson & E. Lawrence, eds., pp. 1:3-1:4, Current Biology Ltd., London, UK, Publisher) and are involved in the allergic response.

Many studies have demonstrated that the extracellular domain of a receptor provides the specific ligand binding characteristic. Furthermore, the cellular environment in which a receptor is expressed may influence the biological response exhibited upon binding of a ligand to the receptor. For example, when a neuronal cell expressing a Trk receptor is exposed to a neurotrophin which binds to that receptor, neuronal survival and differentiation results. When the same receptor is expressed by a fibroblast, exposure to the neurotrophin results in proliferation of the fibroblast (Glass, et al., 1991, Cell 66:405-413).

A class of cell-derived dimeric mitogens with selectivity for vascular endothelial cells has been identified and designated vascular endothelial cell growth factor (VEGF). VEGF has been purified from conditioned growth media of rat glioma cells [Conn et al., (1990), Proc. Natl. Acad. Sci. U.S.A., 87. pp 2628-2632]; and conditioned growth media of bovine pituitary follicle stellate cells [Ferrara and Henzel, (1989), Biochem. Biophys. Res. Comm., 161, pp. 851-858; Gozpadorowicz et al., (1989), Proc. Natl. Acad. Sci. U.S.A., 86, pp. 7311-7315] and conditioned growth medium from human U937 cells [Connolly, D. T. et al. (1989), Science, 246, pp. 1309-1312]. VEGF is a dimer with an apparent molecular mass of about 46 kDa with each subunit having an apparent molecular mass of about 23 kDa. VEGF has some structural similarities to platelet derived growth factor (PDGF), which is a mitogen for connective tissue cells but not mitogenic for vascular endothelial cells from large vessels.

The membrane-bound tyrosine kinase receptor, known as Flt, was shown to be a VEGF receptor [DeVries, C. et al., (1992), Science, 255, pp.989-

991]. The Flt receptor specifically binds VEGF which induces mitogenesis. Another form of the VEGF receptor, designated KDR, is also known to bind VEGF and induce mitogenesis. The partial cDNA sequence and nearly full length protein sequence of KDR is known as well [Terman, B. I. et al., (1991) Oncogene 6, pp. 1677-1683; Terman, B. I. et al., (1992) Biochem. Biophys. Res. Comm. 187, pp. 1579-1586].

Persistent angiogenesis may cause or exacerbate certain diseases such as psoriasis, rheumatoid arthritis, hemangiomas, angiofibromas, diabetic retinopathy and neovascular glaucoma. An inhibitor of VEGF activity would be useful as a treatment for such diseases and other VEGF-induced pathological angiogenesis and vascular permeability conditions, such as tumor vascularization. The present invention relates to a VEGF inhibitor that is based on the VEGF receptor Flt1.

Plasma leakage, a key component of inflammation, occurs in a distinct subset of microvessels. In particular, in most organs plasma leakage occurs specifically in the venules. Unlike arterioles and capillaries, venules become leaky in response to numerous inflammatory mediators including histamine, bradykinin, and serotonin. One characteristic of inflammation is the plasma leakage that results from intercellular gaps that form in the endothelium of venules. Most experimental models of inflammation indicate that these intercellular gaps occur between the endothelial cells of postcapillary and collecting venules (Baluk, P., et al., Am. J. Pathol. 1998 152:1463-76). It has been shown that certain lectins may be used to reveal features of focal sites of plasma leakage, endothelial gaps, and finger-like processes at endothelial cell borders in inflamed venules (Thurston, G., et al., Am. J.

Physiol, 1996, 271: H2547-62). In particular, plant lectins have been used to visualize morphological changes at endothelial cell borders in inflamed venules of, for example, the rat trachea. Lectins, such as conconavalin A and ricin, that bind focally to inflamed venules reveal regions of the subendothelial vessel wall exposed by gaps that correspond to sites of plasma leakage (Thurston, G., et al., Am J Physiol, 1996, 271: H2547-62).

The properties of the microvessels are dynamic. Chronic inflammatory diseases, for example, are associated with microvascular remodeling, including angiogenesis and microvessel enlargement. Microvessels can also remodel by acquiring abnormal phenotypic properties. In a murine model of chronic airway inflammation, airway capillaries acquire properties of venules, including widened vessel diameter, increased immunoreactivity for von Willebrand factor, and increased immunoreactivity for P-selectin. In addition, these remodeled vessels leak in response to inflammatory mediators, whereas vessels in the same position in the airways of normal mice do not.

Certain substances have been shown to decrease or inhibit vascular permeability and/or plasma leakage. For example, mystixins are synthetic polypeptides that have been reported to inhibit plasma leakage without blocking endothelial gap formation (Baluk, P., et al., J. Pharmacol. Exp. Ther., 1998, 284: 693-9). Also, the beta 2-adrenergic receptor agonist formoterol reduces microvascular leakage by inhibiting endothelial gap formation (Baluk, P. and McDonald, D.M., Am. J. Physiol., 1994, 266:L461-8).

The angiopoietins and members of the vascular endothelial growth factor (VEGF) family are the only growth factors thought to be largely specific for vascular endothelial cells. Targeted gene inactivation studies in mice have shown that VEGF is necessary for the early stages of vascular development and that Ang-1 is required for later stages of vascular remodeling.

US Patent No. 6,011,003, issued January 4, 2000, in the name of Metris Therapeutics Limited, discloses an altered, soluble form of FLT polypeptide being capable of binding to VEGF and thereby exerting an inhibitory effect thereon, the polypeptide comprising five or fewer complete immunoglobulin domains.

US Patent No. 5,712,380, issued January 27, 1998 and assigned to Merck & Co., discloses vascular endothelial cell growth factor (VEGF) inhibitors that are naturally occurring or recombinantly engineered soluble forms with or without a C-terminal transmembrane region of the receptor for VEGF.

Also assigned to Merck & Co. is PCT Publication No. WO 98/13071, published April 2, 1998, which discloses gene therapy methodology for inhibition of primary tumor growth and metastasis by gene transfer of a nucleotide sequence encoding a soluble receptor protein which binds to VEGF.

PCT Publication No. WO 97/44453, published November 27, 1997, in the name of Genentech, Inc., discloses novel chimeric VEGF receptor proteins comprising amino acid sequences derived from the vascular

endothelial growth factor (VEGF) receptors Flt1 and KDR, including the *murine homologue* to the human KDR receptor FLK1, wherein said chimeric VEGF receptor proteins bind to VEGF and antagonize the endothelial cell proliferative and angiogenic activity thereof.

PCT Publication No. WO 97/13787, published April 17, 1997, in the name of Toa Gosei Co., LTD., discloses a low molecular weight VEGF inhibitor usable in the treatment of diseases accompanied by neovascularization such as solid tumors. A polypeptide containing the first immunoglobulin-like domain and the second immunoglobulin-like domain in the extracellular region of a VEGF receptor FLT but not containing the sixth immunoglobulin-like domain and the seventh immunoglobulin-like domain thereof shows a VEGF inhibitory activity.

Sharifi, J. et al., 1998, The Quarterly Jour. of Nucl. Med. 42:242-249, disclose that because monoclonal antibodies (MAbs) are basic, positively charged proteins, and mammalian cells are negatively charged, the electrostatic interactions between the two can create higher levels of background binding resulting in low tumor to normal organ ratios. To overcome this effect, the investigators attempted to improve MAb clearance by using various methods such as secondary agents as well as chemical and charge modifications of the MAb itself.

Jensen-Pippo, et al., 1996, Pharmaceutical Research 13:102-107, disclose that pegylation of a therapeutic protein, recombinant human granulocyte colony stimulating factor (PEG-G-CSF), results in an increase in stability and in retention of *in vivo* bioactivity when administered by the intraduodenal route.

Tsutsumi, *et al.*, 1997, Thromb Haemost. 77:168-73, disclose experiments wherein the *in vivo* thrombopoietic activity of polyethylene glycol-modified interleukin-6 (MPEG-IL-6), in which 54% of the 14 lysine amino groups of IL-6 were coupled with PEG, was compared to that of native IL-6.

Yang, *et al.*, 1995, Cancer 76:687-94, disclose that conjugation of polyethylene glycol to recombinant human interleukin-2 (IL-2) results in a compound, polyethylene glycol-modified IL-2 (PEG-IL-2) that retains the in vitro and in vivo activity of IL-2, but exhibits a markedly prolonged circulating half-life.

R. Duncan and F. Spreafico, Clin. Pharmacokinet. 27: 290-306, 296 (1994) review efforts to improve the plasma half-life of asparaginase by conjugating polyethylene glycol.

PCT International Publication No. WO 99/03996 published January 28, 1999 in the name of Regeneron Pharmaceuticals, Inc. and The Regents of The University of California describes modified human noggin polypeptides having deletions of regions of basic amino acids. The modified human noggin polypeptides are described as retaining biological activity while having reduced affinity for heparin and superior pharmacokinetics in animal sera as compared to the unmodified human noggin.

SUMMARY OF THE INVENTION

The present invention is directed to VEGF antagonists with improved pharmacokinetic properties. A preferred embodiment is an isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a VEGF polypeptide comprising (a) a nucleotide sequence encoding a VEGF receptor component operatively linked to (b) a nucleotide sequence encoding a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component of the fusion polypeptide and wherein the nucleotide sequence of (a) consists essentially of a nucleotide sequence encoding the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and a nucleotide sequence encoding the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor.

In a further embodiment, the isolated nucleic acid of the first VEGF receptor is Flt1.

In a further embodiment, the isolated nucleic acid of the second VEGF receptor is Flk1.

In yet another embodiment, the isolated nucleic acid of the second VEGF receptor is Flt4.

In another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

In still another preferred embodiment, the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

In a preferred embodiment of the invention, the multimerizing component comprises an immunoglobulin domain.

In another embodiment, the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include an isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists of a nucleotide sequence selected from the group consisting of

(a) the nucleotide sequence set forth in Figure 13A-13D;
(b) the nucleotide sequence set forth in Figure 14A-14C;
(c) the nucleotide sequence set forth in Figure 15A-15C;
(d) the nucleotide sequence set forth in Figure 16A-16D;
(e) the nucleotide sequence set forth in Figure 21A-21C;
(f) the nucleotide sequence set forth in Figure 22A-22C;
(g) the nucleotide sequence set forth in Figure 24A-24C; and
(h) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c), (d), (e), (f), or (g) and which encodes a fusion polypeptide molecule having

the biological activity of the modified Flt1 receptor fusion polypeptide.

In a further embodiment of the invention, a fusion polypeptide is encoded by the isolated nucleic acid molecules described above.

A preferred embodiment is a composition capable of binding a VEGF molecule to form a nonfunctional complex comprising a multimer of the fusion polypeptide.

Also preferred is a composition wherein the multimer is a dimer.

In yet another embodiment, the composition is in a carrier.

Another embodiment is a vector which comprises the nucleic acid molecules described above, including an expression vector comprising a the nucleic acid molecules described wherein the nucleic acid molecule is operatively linked to an expression control sequence.

Other included embodiments are a host-vector system for the production of a fusion polypeptide which comprises the expression vector, in a suitable host cell; the host-vector system wherein the suitable host cell is a bacterial cell, yeast cell, insect cell, or mammalian cell; the host-vector system wherein the suitable host cell is E. Coli; the host-vector system wherein the suitable host cell is a COS cell; the host-vector system wherein the suitable host cell is a CHO cell.

Another embodiment of the invention is a method of producing a fusion polypeptide which comprises growing cells of the host-vector system under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

Additional embodiments include a fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D or Figure 24A-24C, which has been modified by acetylation or pegylation wherein the acetylation is accomplished with at least about a 100 fold molar excess of acetylation reagent or wherein acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess or wherein the pegylation is 10K or 20K PEG.

A preferred embodiment includes a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above, including embodiments wherein the mammal is a human, the fusion polypeptide is acetylated or the fusion polypeptide is pegylated.

A further embodiments is a fusion polypeptide which specifically binds the VEGF receptor ligand VEGF.

A preferred embodiment of the invention is a method of blocking blood vessel growth in a human comprising administering an effective amount of the fusion polypeptide described above.

Also preferred is a method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide described above.

Preferred embodiments of these methods are wherein the mammal is a human.

Further embodiments of the methods of the invention include attenuation or prevention of tumor growth in a human; attenuation or prevention of edema in a human, especially wherein the edema is brain edema; attenuation or prevention of ascites formation in a human, especially wherein the ascites is ovarian cancer-associated ascites.

Preferred embodiments of the invention include a fusion polypeptide capable of binding a VEGF polypeptide comprising (a) a VEGF receptor component operatively linked to (b) a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component in the fusion polypeptide and consists essentially of the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor.

In a further embodiment of the fusion polypeptide, the first VEGF receptor is Flt1.

In yet a further embodiment of the fusion polypeptide, the second VEGF receptor is Flk1.

Still another embodiment of the fusion polypeptide is one in which the second VEGF receptor is Flt4.

Preferred embodiments include a fusion polypeptide wherein amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor and a fusion polypeptide wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

In yet another embodiment, the fusion polypeptide multimerizing component comprises an immunoglobulin domain including an embodiment wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

Preferred embodiments include a fusion polypeptide comprising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence selected from the group consisting of (a) the amino acid sequence set forth in Figure 13A-13D; (b) the amino acid sequence set forth in Figure 14A-14C; (c) the amino acid sequence set forth in Figure 15A-15C; (d) the amino acid sequence set forth in Figure 16A-16D; (e) the amino acid sequence set forth in Figure 21A-21C; (f) the amino acid sequence set forth in Figure 22A-22C; and (g) the amino acid sequence set forth in Figure 24A-24C.

Another preferred embodiment is a method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal the fusion polypeptide described above.

An alternative preferred embodiment is a method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide described above.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. IEF gel analysis of unmodified and acetylated Flt1(1-3)-Fc proteins. Unmodified Flt1(1-3)-Fc protein is unable to enter the gel due to its >9.3 pI, whereas acetylated Flt1(1-3)-Fc is able to enter the gel and equilibrate at pI 5.2.

Figure 2. Binding of unmodified Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc proteins to Matrigel® coated plates. Unmodified Flt1(1-3)-Fc proteins binds extensive to extracellular matrix components in Matrigel®, whereas acetylated Flt1(1-3)-Fc does not bind.

Figure 3. Binding of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc in a Biacore-based assay. Acetylated (columns 13-16), pegylated (columns 17-20), and heparin-treated Flt1(1-3)-Fc (columns 21-24) are each able to completely compete with the Biacore chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control (columns 1-4) and irrelevant protein (columns 5-8). Unmodified Flt1(1-3)-Fc (columns 5-6) appears to only partially compete with Biacore chip-bound Flt1(1-3)-Fc for VEGF binding.

However, washing the bound samples with 0.5M NaCl (columns 7-8) results in a binding profile similar to the modified forms of Flt1(1-3)-Fc, indicating that the unmodified protein is exhibiting non-specific binding to the chip that can be eliminated by the salt wash.

Figure 4. Binding of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc to VEGF in an ELISA-based assay. Both pegylated and acetylated Flt1(1-3)-Fc proteins bind to VEGF with affinities approaching that of unmodified Flt1(1-3)-Fc.

Figure 5. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc. Balb/c mice (23-28g) were injected subcutaneously with 4mg/kg of unmodified, acetylated, or pegylated Flt1(1-3)-Fc. The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, and 3 days after injection of protein and the sera were assayed in a standard ELISA-based assay designed to detect Flt1(1-3)-Fc protein. The T_{max} for all of the Flt1(1-3)-Fc proteins was between the 6 hour and 24 hour time points. The C_{max} for the different proteins was as follows: Unmodified: 0.06 μg/ml - 0.15 μg/ml; acetylated: 1.5 μg/ml - 4.0 μg/ml; and pegylated: approximately 5 μg/ml.

Figure 6A-6B. IEF gel analysis of unmodified and step-acetylated Flt1(1-3)-Fc proteins. Unmodified Flt1(1-3)-Fc protein is unable to enter the gel due to its >9.3 pI, whereas most of the step-acetylated Flt1(1-3)-Fc samples (30-100 fold excess samples) were able to migrate into the gel and equilibrate at pIs ranging between 4.55 - 8.43, depending on the degree of acetylation.

Figure 7. Binding of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc proteins to Matrigel® coated plates. As with the irrelevant control protein, rTie2-Fc, step-acetylated Flt1(1-3)-Fc (20 and 30 fold excess samples) does not exhibit any binding to the Matrigel coated plate, whereas the non-acetylated Flt1(1-3)-Fc protein exhibits significant binding. The 10 fold excess sample shows reduced binding, but the degree of acetylation is not enough to completely block binding to extracellular matrix components.

Figure 8. Binding of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc in a Biacore-based assay. At a sub-stoichiometric ratio (0.5 μg/ml of either unmodified Flt1(1-3) or step-acetylated Flt1(1-3)-Fc vs. 0.2 μg/ml VEGF), there is not enough Flt1(1-3)-Fc (either unmodified or step-acetylated) in the solution to completely bind the VEGF. At 1.0 μg/ml, which approximates a 1:1 stoichiometric ratio, the both unmodified and step-acetylated Flt1(1-3)-Fc are better able to compete for VEGF binding, but there is still insufficient Flt1(1-3)-Fc protein (either unmodified or step-acetylated) to completely saturate the available VEGF. However, at 5.0 μg/ml, which is several times greater than a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to saturate the VEGF, regardless of the degree of acetylation.

Figure 9. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc. Balb/c mice (23-28g) were injected subcutaneously with 4mg/kg of unmodified or 10, 20, 40, 60 and 100 fold excess samples of step-acetylated Flt1(1-3)-Fc (3 mice for

unmodified, 10, 20 and 40 fold excess samples and 2 mice for 60 and 100 fold excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1(1-3)-Fc. The T_{max} for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the C_{max} was as follows: Unmodified Flt1(1-3)-Fc: 0.06μg/ml; 10 fold excess sample: - 0.7μg/ml, 20 fold excess sample - 2μg/ml, 40 fold excess sample - 4μg/ml, 60 fold excess sample - 2μg/ml, 100 fold excess sample - 1μg/ml.

Figure 10A-10D. Nucleic acid and deduced amino acid sequence of Flt1(1-3)-Fc.

Figure 11. Schematic diagram of the structure of Flt1.

Figure 12A and 12B. Hydrophilicity analysis of the amino acid sequences of Ig domain 2 and Ig domain 3 of Flt1.

Figure 13A-13D. Nucleic acid and deduced amino acid sequence of Mut1: Flt1(1-$3_{\Delta B}$)-Fc.

Figure 14A-14 C. Nucleic acid and deduced amino acid sequence of Mut2: Flt1(2-$3_{\Delta B}$)-Fc.

Figure 15A-15C. Nucleic acid and deduced amino acid sequence of Mut3: Flt1(2-3)-Fc.

Figure 16A-16D. Nucleic acid and deduced amino acid sequence of Mut4: Flt1(1-$3_{R->N}$)-Fc.

Figure 17. Binding of unmodified Flt1(1-3)-Fc, basic region deletion mutant Flt1(1-3)-Fc, and Flt1(1-$3)_{R->N}$ mutant proteins in a Biacore-based assay. At the sub-stoichiometric ratio (0.25 μg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. μg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF to the Flt1(1-3)-Fc immobilized on the Biacore chip. At 0.5 μg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the Biacore chip. At 1.0 μg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, which is approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the Biacore chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-$3_{\Delta B}$)-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1(1-$3_{R->N}$)-Fc is somewhat less efficient at blocking binding

Figure 18. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-$3_{\Delta B}$)-Fc, Mut2: Flt1(2-$3_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins to Matrigel® coated plates. Unmodified Flt1(1-3)-Fc protein binds avidly to these wells, the Mut3: Flt1(2-3)-Fc protein binds somewhat more weakly, the Mut1: Flt1(1-$3_{\Delta B}$)-Fc protein binds more weakly still, and the Mut2: Flt1(2-$3_{\Delta B}$)-Fc protein shows the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1(1-$3_{R->N}$)-Fc

glycosylation mutant protein shows only marginal benefit on the Matrigel assay.

Figure 19. Binding of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-$3_{\Delta B}$)-Fc, Mut2: Flt1(2-$3_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins in an ELISA-based assay. At the concentrations tested, unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-$3_{\Delta B}$)-Fc, Mut2: Flt1(2-$3_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins bind VEGF similarly.

Figure 20. Pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-$3_{\Delta B}$)-Fc, Mut2: Flt1(2-$3_{\Delta B}$)-Fc, and Flt1(2-3) mutant proteins. the Cmax for these reagents was as follows: Unmodified Flt1(1-3)-Fc - 0.15μg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc - 1.5μg/ml; and Mut1: Flt1(1-$3_{\Delta B}$)-Fc - 0.7μg/ml.

Figure 21A-21C. Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.Flk1D3.FcΔC1(a).

Figure 22A-22C. Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed Flt1D2.VEGFR3D3.FcΔC1(a).

Figure 23. Extracellular Matrix (ECM) Assay. The results of this assay demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

Figure 24A-24C. Nucleotide and deduced amino acid sequence of the modified Flt1 receptor termed VEGFR1R2-FcΔC1(a).

Figure 25A-25C. Phosphorylation assay. At a 1.5 molar excess of either Flt1(1-3)-Fc , Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.FcΔC1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.FcΔC1(a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similar results are seen in Figure 25B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In Figure 25C, where the modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.FcΔC1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

Figure 26A-26B. Phosphorylation assay. Detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are not phosphorylated by challenge samples which have VEGF165 preincubated with 1 and 2 fold molar excess (Figure 26A) or 3 and 4 fold molar excess (Figure 26B) of either transient Flt1D2Flk1D3.FcΔC1(a), stable Flt1D2Flk1D3.FcΔC1(a), or transient VEGFR1R2-FcΔC1(a). At all modified Flt1 receptor concentrations tested there is complete binding of VEGF165 ligand

during the preincubation, resulting in no detectable stimulation of cell-surface receptors by unbound VEGF165 as compared to control media challenge.

Figure 27. MG/R2 Cell proliferation assay. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a), plus an irrelevant receptor termed Tie2-Fc as a negative control, were titrated from 40nM to 20pM and incubated on the cells for 1hr at 37°C. Human recombinant VEGF165 in defined media was then added to all the wells at a concentration of 1.56nM. The negative control receptor Tie2-Fc does not block VEGF165-induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.FcΔC1(a) blocks 1.56nM VEGF165 with a half maximal dose of 0.8nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.FcΔC1(a) are less effective in blocking VEGF165 in this assay with a half maximal dose of ~ 2nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units.

Figure 28. Biacore analysis of Binding Stoichiometry. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) molecule.

Figure 29 and Figure 30. Size Exclusion Chromatography Stoichiometry. Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) at a concentration of 1nM (estimated to be 1000 times higher than the KD of the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a)/VEGF165 interaction) were mixed with varied concentrations of VEGF165. After incubation, concentrations of the free Flt1D2Flk1D3.FcΔC1(a) in solution were measured. The data shows that the addition of 1 nM VEGF165 into the Flt1D2Flk1D3.FcΔC1(a) solution completely blocks Flt1D2Flk1D3.FcΔC1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.FcΔC1(a) molecule.

Figure 31. Size Exclusion Chromatography (SEC) under native conditions. Peak #1 represents the Flt1D2Flk1D3.FcΔC1(a)/ VEGF165 complex and peak #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2 ml were combined and guanidinium hydrochloride (GuHCl)was added to a final concentration 4.5M to dissociate the complex.

Figure 32. Size Exclusion Chromatography (SEC) under dissociative conditions. To separate the components of the receptor-ligand complex and to determine their molar ratio, 50µl of dissociated complex was loaded onto a Superose 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted. Peak #1 represents Flt1D2Flk1D3.FcΔC1(a) and peak #2 represents VEGF165.

Figure 33, Figure 34 and Figure 35. Size Exclusion Chromatography (SEC) with On-Line Light Scattering. Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa Barbara, California) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. As shown in Figure 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300 (Figure 33), the MW of VEGF165 at the peak position is 44 390 (Figure 34) and the MW of R1R2 at the peak is 113 300 (Figure 35).

Figure 36. Peptide mapping and glycosylation analysis. The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 is disulfide bonded to Cys76. Cys121 is disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, it can not be determined

whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is disulfide bonded to Cys306. Cys 352 is disulfide bonded to Cys410.

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) and are found to be glycosylated to varying degrees. Complete glycosylation is observed at Asn33, Asn193, and Asn282. Partial glycosylation is observed on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the Figure.

Figure 37. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a). Balb/c mice were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a), CHO stably expressed Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently expressed VEGFR1R2-FcΔC1(a). The mice were tail bled at 1, 2, 4, 6, 24 hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a). The Tmax for Flt1(1-3)-Fc (A40) was at 6 hrs while the Tmax for the transient and stable Flt1D2.Flk1D3.FcΔC1(a) and the transient VEGFR1R2-FcΔC1(a) was 24hrs. The Cmax for Flt1(1-3)-Fc (A40) was 8μg/ml, For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the Cmax was 18μg/ml and the Cmax for the stable VEGFR1R2-FcΔC1(a) was 30μg/ml.

Figure 38. Pharmacokinetics of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). Balb/c mice were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.FcΔC1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5 whereas Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) were detectable for 15 days or more.

Figure 39. The Ability of Flt1D2.Flk1D3.FcΔC1(a) to Inhibit HT-1080 Fibrosarcoma Tumor Growth In Vivo. Every other day or 2 times per week treatment of SCID mice with Flt1D2.Flk1D3.FcΔC1(a) at 25mg/Kg significantly decreases the growth of subcutaneous HT-1080 fibrosarcoma tumors.

Figure 40. The Ability of Flt1D2.Flk1D3.FcΔC1(a) to Inhibit C6 Glioma Tumor Growth In Vivo. Every other day or 2 times a week treatment of SCID mice with Flt1D2.Flk1D3.FcΔC1(a) significantly decreases the growth of subcutaneous C6 glioma tumors at doses as low as 2.5mg/Kg.

Figure 41. VEGF-Induced Uterine Hyperpermeability. PMSG injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats

results in a surge of estradiol after 2 days which in turn causes an induction of VEGF in the uterus. This induction results in hyperpermeability of the uterus and an increase in uterine wet. Subcutaneous injection of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) at 25mg/kg at 1hr after PMSG injection results in about a 50% inhibition of the increase in uterine wet weight.

Figure 42A-42B. Assessment of Corpus Luteum Angiogenesis Using Progesterone as a Readout. PMSG was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats, resulting in a fully functioning corpus luteum containing a dense network of blood vessels that secretes progesterone into the blood stream to prepare the uterus for implantation. The induction of angiogenesis in the corpus luteum requires VEGF. Resting levels of progesterone are about 5ng/ml and can be induced to 25-40ng/ml after PMSG. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2.Flk1D3.FcΔC1(a) at 25mg/kg or 5mg/kg at 1hr. after PMSG injection resulted in a complete inhibition of the progesterone induction on day 4.

DETAILED DESCRIPTION OF THE INVENTION

It has been a long standing problem in the art to produce a receptor based VEGF antagonist that has a pharmacokinetic profile that is appropriate for consideration of the antagonist as a therapeutic candidate. Applicants describe herein, for the first time, a chimeric

polypeptide molecule, capable of antagonizing VEGF activity, that exhibits improved pharmacokinetic properties as compared to other known receptor-based VEGF antagonists. The chimeric polypeptide molecules described herein thus provide for the first time appropriate molecules for use in therapies in which antagonism of VEGF is a desired result.

The present invention provides for novel chimeric polypeptide molecules formed by fusing a modified extracellular ligand binding domain of the Flt1 receptor to the Fc region of IgG.

The extracellular ligand binding domain is defined as the portion of a receptor that, in its native conformation in the cell membrane, is oriented extracellularly where it can contact with its cognate ligand. The extracellular ligand binding domain does not include the hydrophobic amino acids associated with the receptor's transmembrane domain or any amino acids associated with the receptor's intracellular domain. Generally, the intracellular or cytoplasmic domain of a receptor is usually composed of positively charged or polar amino acids (i.e. lysine, arginine, histidine, glutamic acid, aspartic acid). The preceding 15-30, predominantly hydrophobic or apolar amino acids (i.e. leucine, valine, isoleucine, and phenylalanine) comprise the transmembrane domain. The extracellular domain comprises the amino acids that precede the hydrophobic transmembrane stretch of amino acids. Usually the transmembrane domain is flanked by positively

charged or polar amino acids such as lysine or arginine. von Heijne has published detailed rules that are commonly referred to by skilled artisans when determining which amino acids of a given receptor belong to the extracellular, transmembrane, or intracellular domains (See von Heijne, 1995, BioEssays 17:25-30). Alternatively, websites on the Internet, such as http://ulrec3.unil.ch/software/TMPRED_form.html. have become available to provide protein chemists with information about making predictions about protein domains.

The present invention provides for the construction of nucleic acid molecules encoding chimeric polypeptide molecules that are inserted into a vector that is able to express the chimeric polypeptide molecules when introduced into an appropriate host cell. Appropriate host cells include, but are not limited to, bacterial cells, yeast cells, insect cells, and mammalian cells. Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the chimeric polypeptide molecules under control of transcriptional/translational control signals. These methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination) (See Sambrook, et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY).

Expression of nucleic acid molecules encoding the chimeric polypeptide molecules may be regulated by a second nucleic acid sequence so that the chimeric polypeptide molecule is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the chimeric polypeptide molecules described herein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control expression of the chimeric polypeptide molecules include, but are not limited to, the long terminal repeat as described in Squinto et al., (1991, Cell 65:1-20); the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the CMV promoter, the M-MuLV 5' terminal repeat the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothionine gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25, see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADH (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and

have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al, 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94); myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Shani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Thus, according to the invention, expression vectors capable of being replicated in a bacterial or eukaryotic host comprising chimeric polypeptide molecule-encoding nucleic acid as described herein, are used to transfect the host and thereby direct expression of such nucleic acids to produce the chimeric polypeptide molecules, which may then be recovered in a biologically active form. As used herein, a biologically active form includes a form capable of binding to VEGF.

Expression vectors containing the chimeric nucleic acid molecules described herein can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization using probes comprising sequences that are homologous to the inserted chimeric polypeptide molecule sequences. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the chimeric polypeptide molecule DNA sequence is inserted within the marker gene sequence of the vector, recombinants containing the insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying

the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the chimeric polypeptide molecules.

Cells of the present invention may transiently or, preferably, constitutively and permanently express the chimeric polypeptide molecules.

The chimeric polypeptide molecules may be purified by any technique which allows for the subsequent formation of a stable, biologically active chimeric polypeptide molecule. For example, and not by way of limitation, the factors may be recovered from cells either as soluble proteins or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis (see, for example, Builder, et al., US Patent No. 5,663,304). In order to further purify the factors, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used.

In one embodiment of the invention, the nucleotide sequence encoding the first component is upstream of the nucleotide sequence encoding the second component. In another embodiment of the invention, the nucleotide sequence encoding the first component is downstream of the nucleotide sequence encoding the second component. Further embodiments of the invention may be prepared in which the order of the

first, second and third fusion polypeptide components are rearranged. For example, if the nucleotide sequence encoding the first component is designated 1, the nucleotide sequence encoding the second component is designated 2, and the nucleotide sequence of the third component is designated 3, then the order of the components in the isolated nucleic acid of the invention as read from 5' to 3' may be any of the following six combinations: 1,2,3; 1,3,2; 2,1,3; 2,3,1; 3,1,2; or 3,2,1.

The present invention also has diagnostic and therapeutic utilities. In particular embodiments of the invention, methods of detecting aberrancies in the function or expression of the chimeric polypeptide molecules described herein may be used in the diagnosis of disorders. In other embodiments, manipulation of the chimeric polypeptide molecules or agonists or antagonists which bind the chimeric polypeptide molecules may be used in the treatment of diseases. In further embodiments, the chimeric polypeptide molecule is utilized as an agent to block the binding of a binding agent to its target.

By way of example, but not limitation, the method of the invention may be useful in treating clinical conditions that are characterized by vascular permeability, edema or inflammation such as brain edema associated with injury, stroke or tumor; edema associated with inflammatory disorders such as psoriasis or arthritis, including rheumatoid arthritis; asthma; generalized edema associated with burns; ascites and pleural effusion associated with tumors, inflammation or

trauma; chronic airway inflammation; capillary leak syndrome; sepsis; kidney disease associated with increased leakage of protein; and eye disorders such as age related macular degeneration and diabetic retinopathy.

An amino acid sequence analysis of Flt1(1-3)-Fc revealed the presence of an unusually high number (46) of the basic amino acid residue lysine. An IEF analysis of Flt1(1-3)-Fc showed that this protein has pI greater than 9.3, confirming the prediction that the protein is very basic. It was hypothesized that the basic nature of Flt1(1-3)-Fc protein was causing it to bind to extracellular matrix components and that this interaction might be the cause of the extremely short detectable circulating serum half-life exhibited by Flt1(1-3)-Fc when injected into mice. In order to test this hypothesis, Flt1(1-3)-Fc protein was acetylated at the lysine residues to reduce the basic charge. Acetylated Flt1(1-3)-Fc was then tested in the assays described *infra*.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Example 1: Expression of Flt1(1-3)-Fc protein in CHO K1 cells.

Using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor

Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY), the gene encoding Flt1(1-3)-Fc was inserted into the expression vector pEE14.1 (Lonza Biologics, plc) at a multiple cloning site downstream of the CMV promoter. CHO K1 cells were transfected with the pEE14.1/Flt1(1-3)-Fc DNA construct using lipofectamine (Gaithersburg, MD). The transfected CHO K1 cells were grown in glutamine-free DMEM (JRH, Kansas City, MO) containing 25μM methionine sulfoximine (MSX) from Sigma Inc., St. Louis, MO, and high recombinant protein expressors were obtained by screening the CHO K1 cell supernatants from over 100 hand-picked colony isolates using a standard immunoassay which captures and detects human Fc. The selected hand-picked clone was amplified in the presence of 100 μM MSX followed by a second round of screening of the amplified clones. The highest producing clone had a specific productivity of recombinant Flt1(1-3)-Fc protein of 55 pg/cell/day.

The selected clone was expanded in 225cm^2 T-flasks (Corning, Acton, MA) and then into 8.5L roller bottles (Corning, Acton, MA) using the cell culture media described *supra*. Cells were removed from the roller bottles by standard trypsinization and put into 3.5L of suspension medium. The suspension medium is comprised of glutamine-free ISCHO medium (Irvine Scientific, Santa Ana, CA) containing 5% fetal bovine serum (FBS from Hyclone Labs, Logan, UT), 100μM MSX and GS supplement (JRH Scientific, Kansas City, MO) in a 5L Celligen bioreactor (New Brunswick Scientific, New Brunswick, NJ) at a density

of 0.3 x 10^6 cells/mL. After the cells reached a density of 3.6 x 10^6/mL and were adapted to suspension they were transferred to a 60L bioreactor (ABEC, Allentown, PA) at a density of 0.5 x 10^6 cells/mL in 20L of ISCHO medium with 5% fetal bovine serum. After two days an additional 20L of ISCHO + 5% fetal bovine serum was added to the bioreactor. The cells were allowed to grow for an additional two days reaching a final density of 3.1 x 10^6 cells/mL, and a final Flt1(1-3)-Fc concentration at harvest was 95 mg/L. At harvest the cells were removed by tangential flow filtration using 0.45μm Prostak Filters (Millipore, Inc., Bedford, MA).

Example 2: Purification of Flt1(1-3)-Fc protein obtained from CHO K1 cells

Flt1(1-3)-Fc protein was initially purified by affinity chromatography. A Protein A column was used to bind, with high specificity, the Fc portion of the molecule. This affinity-purified protein was then concentrated and passed over a SEC column. The protein was then eluted into the formulation buffer. The following describes these procedures in detail.

Materials and Methods

All chemicals were obtained from J.T. Baker, Phillipsburg, NJ with the exception of PBS; which was obtained as a 10X concentrate from Life

Technologies, Gaithersburg, MD. Protein A Fast Flow and Superdex 200 preparation grade resins were obtained from Pharmacia, Piscataway, NJ. Equipment and membranes for protein concentration were obtained from Millipore, Bedford, MA.

Approximately 40L of 0.45µm-filtered CHO conditioned media containing Flt1(1-3)-Fc protein was applied to a 290mL Protein A Fast Flow column (10cm diameter) that had been equilibrated with PBS. The column was washed with PBS containing 350mM NaCl and 0.02% CHAPS and the bound protein was eluted with 20mM Citric Acid containing 10mM Na_2HPO_4. The single peak in the elution was collected and its pH was raised to neutrality with 1M NaOH. The eluate fractions was concentrated to approximately 9 mg/mL using 10K regenerated cellulose membranes by both tangential flow filtration and by stirred cell concentration. To remove aggregates and other contaminants, the concentrated protein was applied to a column packed with Superdex 200 preparation grade resin (10cm x 55cm) and run in PBS containing 5 % glycerol. The main peak fractions were pooled, sterile filtered, aliquoted and stored at -80°C.

Example 3: Acetylation of Flt1(1-3)-Fc protein.

Two milligrams of Flt1(1-3)-Fc protein were acetylated as described in the instruction manual provided with the sulfo-NHS-acetate modification kit (Pierce Chemical Co., Rockford, IL, Cat.#26777).

Example 4: Characterization of acetylated Flt1(1-3)-Fc protein.

(a.) IEF analysis: Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc were analyzed by standard IEF analysis. As shown in Figure 1, Flt1(1-3)-Fc protein is not able to migrate into the gel and therefore must have a pI greater than 9.3, the highest pI in the standard. However, acetylated Flt1(1-3)-Fc is able to migrate into the gel and equilibrate at a pI of approximately 5.2. This result demonstrates that acetylation reduces the net positive charge of the protein and therefore its pI considerably.

(b.) Binding to extracellular matrix components

To test for binding to extracellular matrix components, Flt1(1-3)-Fc and acetylated Flt1(1-3)-Fc where tested in an assay designed to mimic the interaction with extracellular matrix components. In this assay, 96-well tissue culture plates are coated with Matrigel (Biocoat MATRIGEL® matrix thin layer 96 well plate, Catalog #40607, Becton Dickinson Labware, Bedford, MA). The plates are incubated with varying concentrations of either Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, or rTie2-Fc (an irrelevant control) protein are added to the wells. The plates are incubated for 1-2 hours at either room temperature or 37°C degrees and then detection of bound proteins is accomplished by adding a secondary alkaline phosphatase-conjugated anti-human Fc antibody to

the wells. Finally, alkaline phosphatase substrate is added to the wells and optical density is measured. Figure 2 shows the results of this assay. Like the irrelevant control protein rTie2-Fc, acetylated Flt1(1-3)-Fc does not exhibit any binding to the Matrigel coated plate, whereas the non-acetylated Flt1(1-3)-Fc protein exhibits significant binding. This result indicates that acetylation of basic amino acid residues is an effective way to interfere with the charge interactions that exist between positively charged proteins and the negatively charged extracellular matrix components they are exposed to *in vivo*.

Example 5: Pegylation of Flt1(1-3)-Fc protein.

Although pegylation (polyethylene glycol - PEG) of proteins has been shown to increase their *in vivo* potency by enhancing stability and bioavailability while minimizing immunogenicity (see references cited *supra*), it is counter-intuitive that pegylating molecules that are too large to be filtered by the kidney glomeruli would improve their pharmacokinetic properties. Without being bound by theory, Applicants postulated that pegylation of the Flt1(1-3)-Fc molecules could improve the pharmacokinetic properties, possibly not by altering the positive charge or by decreasing the pI of Flt1(1-3)-Fc, but rather by physically shielding the positive charges from interacting with the extracellular matrix. Applicants decided to attempt to improve the pharmacokinetic properties of Flt1(1-3)-Fc molecules by attaching strands of 20K PEGs as described *infra*.

Materials and Methods

Purified Flt1(1-3)-Fc derived from CHO cells (see *supra*) was used in the following pegylation experiments. Functionalized PEGs were obtained from Shearwater Polymers, Huntsville, AL; Bicine from Sigma, St Louis, MO; Superose 6 column from Pharmacia, Piscataway, NJ; PBS as a 10X concentrate from Life Technologies, Gaithersburg, MD; Glycerol from J.T. Baker, Phillipsburg, NJ; and Bis-Tris precast gels from Novex, CA.

20K PEG strands functionalized with amine-specific terminal moieties were used in small-scale reaction studies that were set-up to evaluate different reaction conditions in which the PEG:protein stoichiometry was varied. Based on these reactions and the analyses of samples on standard SDS-PAGE, Flt1(1-3)-Fc at a concentration of 1.5 mg/mL was reacted at pH 8.1 with 20K SPA-PEG (PEG succinimidyl propionate) molecules at a PEG-to-Flt1(1-3)-Fc monomer molar ratio of 1:6. The reaction was allowed to proceed at 8°C overnight. For initial purification, the reaction products were applied to a 10mm x 30cm Superose 6 column equilibrated with PBS containing 5% Glycerol. The column appeared to separate pegylated Flt1(1-3)-Fc molecules based on the extent of pegylation. Fractions corresponding to what appeared to be primarily mono-pegylated and di-pegylated dimeric Flt1(1-3)-Fc, as judged by banding patterns on reducing and non-reducing SDS-PAGE gels

were pooled. The protein concentration was determined by measuring absorbance at 280 nm. The pegylated Flt1(1-3)-Fc protein was sterile filtered, aliquoted and stored at -40°C.

Example 6: Binding of unmodified, acetylated, and pegylated Flt1(1-3)-Fc in a Biacore-based assay.

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a Biacore-based assay to evaluate their ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein was immobilized on the surface of a Biacore chip (see Biacore Instruction Manual, Pharmacia, Inc., Piscataway, NJ, for standard procedures) and a sample containing 0.2 μg/ml VEGF and either unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc or pegylated Flt1(1-3)-Fc (each at 25 μg/ml) was passed over the Flt1(1-3)-Fc-coated chip. To minimize the effects of non-specific binding, the bound samples were washed with a 0.5M NaCl wash. In one sample, unmodified Flt1(1-3)-Fc was mixed with heparin. Heparin is a negatively charged molecule and the Flt1(1-3)-Fc protein is a positively charged molecule, so when the two molecules are mixed together, they should interact through their respective charges. This essentially neutralizes Flt1(1-3)-Fc's inherent positive charge making the molecule behave as if it has been chemically or genetically modified so as to reduce its charge and its tendency to bind via charge interactions. As shown in Figure 3, acetylated (columns 13-16), pegylated (columns 17-20), and heparin-treated Flt1(1-3)-Fc

(columns 21-24) are each able to completely compete with the Biacore chip-bound Flt1(1-3)-Fc for VEGF binding as compared to control (columns 1-4) and irrelevant protein (columns 5-8). Unmodified Flt1(1-3)-Fc (columns 5-6) appeared to only partially compete with Biacore chip-bound Flt1(1-3)-Fc for VEGF binding. However, washing the bound samples with 0.5M NaCl (columns 7-8) resulted in a binding profile similar to the modified forms of Flt1(1-3)-Fc, indicating that the unmodified protein was exhibiting non-specific binding to the chip that could be eliminated by the salt wash.

Example 7: Binding of unmodified, acetylated, and pegylated Flt1(1-3)-Fc in an ELISA-based assay.

Unmodified, acetylated, and pegylated Flt1(1-3)-Fc proteins were tested in a standard ELISA-based assay to evaluate their ability to bind the Flt1 receptor ligand VEGF. As shown in Figure 4, both pegylated and acetylated Flt1(1-3)-Fc proteins are capable of binding to VEGF, demonstrating that modifying the protein either by pegylation or acetylation does not destroy its ability to bind its ligand.

Example 8: Pharmacokinetic analysis of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and pegylated Flt1(1-3)-Fc.

In vivo experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, acetylated Flt1(1-3)-Fc, and

pegylated Flt1(1-3)-Fc protein. Balb/c mice (23-28g; 3 mice/group) were injected subcutaneously with 4mg/kg of unmodified, acetylated, or pegylated Flt1(1-3)-Fc. The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, and 3 days after injection of protein. The sera were assayed in a standard ELISA-based assay designed to detect Flt1(1-3)-Fc protein. Briefly, the assay involves coating an ELISA plate with VEGF, binding the unmodified, acetylated, or pegylated Flt1(1-3)-Fc-containing sera, and reporting with an anti-Fc antibody linked to alkaline phosphatase. As shown in Figure 5, the Tmax for all of the Flt1(1-3)-Fc proteins was between the 6 hour and 24 hour time points. The Cmax for the different proteins was as follows: Unmodified: 0.06 μ/ml - 0.15 μg/ml; acetylated: 1.5 μg/ml - 4.0 μg/ml; and pegylated: approximately 5 μg/ml.

Example 9: Step-acetylation of Flt1(1-3)-Fc

To determine what minimal amount of acetylation is necessary to eliminate binding to extracellular matrix components, an experiment was designed that acetylated the Flt1(1-3)-Fc protein in a step-wise fashion by using increasing amounts of molar excess of acetylation reagent in the acetylation reaction mixture. The range of molar excess was as follows: 0, 10, 20, 30, 40, 50, 60, 70, 80 , 90, and 100 moles of acetylation reagent per 1 mole of Flt1(1-3)-Fc monomer. The reactions were performed as detailed in the instruction manual provided with the

sulfo-NHS-Acetate modification kit (Pierce Chemical Co., Rockford, IL, Cat.# 26777).

Example 10: Characterization of step-acetylated Flt1(1-3)-Fc.

(a.) IEF analysis Unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc proteins were analyzed by standard IEF analysis. As shown in Figure 6A-6B, unmodified Flt1(1-3)-Fc protein was not able to migrate into the gel due to its extremely high pI (greater than 9.3). However, most of the step-acetylated Flt1(1-3)-Fc samples (30-100 fold molar excess samples) were able to migrate into the gel and equilibrate at pIs ranging between 4.55 - 8.43, depending on the degree of acetylation of the protein. This result demonstrates that acetylation can change the positive charge of the protein in a dose-dependent manner and that reduction of the pI can be controlled by controlling the degree of acetylation.

(b.) Binding of step-acetylated Flt1(1-3)-Fc to extracellular matrix components

To test for binding to extracellular matrix components, Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc where tested in the above-described assay designed to mimic the interaction with extracellular matrix components. Varying concentrations of either unmodified Flt1(1-3)-Fc,

step-acetylated Flt1(1-3)-Fc (10, 20, and 30 fold molar excess samples), or rTie2-Fc (an irrelevant control) protein were added to the wells. The plates were incubated for 1-2 hours at room temperature or 37°C and then detection of bound proteins was accomplished by adding a secondary alkaline phosphatase-conjugated anti-human Fc antibody to the wells. Alkaline phosphatase substrate was subsequently added to the wells and optical density measured. Figure 7 shows the results of this assay. Like the irrelevant control protein rTie2-Fc, step-acetylated Flt1(1-3)-Fc (20 and 30 fold molar excess samples) did not exhibit any significant binding to the Matrigel coated plate, whereas the non-acetylated Flt1(1-3)-Fc protein exhibited significant binding. The binding is saturable, indicating that the Flt1(1-3)-Fc protein may be binding to specific sites, rather than a more general charge-mediated interaction that might not be saturable. The 10 fold molar excess sample showed reduced binding, but the degree of acetylation was not enough to completely block binding to extracellular matrix components. The 20 fold molar excess and higher samples displayed no detectable binding, despite the fact that by IEF analysis (Figure 6A and 6B) the lower molar excess samples still had a large net positive charge. This result demonstrates that it is not necessary to completely acetylate all available basic amino acids in order to eliminate binding to extracellular matrix components.

(c.) Binding of step-acetylated Flt1(1-3)-Fc in a Biacore-based assay.

Unmodified and step-acetylated Flt1(1-3)-Fc proteins where tested in a Biacore-based assay to evaluate their ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein (0.5, 1.0, or 5.0 μg/ml) was immobilized on the surface of a Biacore chip (see Biacore Instruction Manual, Pharmacia, Inc., Piscataway, NJ, for standard procedures) and a solution containing 0.2 μg/ml VEGF and either unmodified Flt1(1-3)-Fc (at either 0.5, 1.0, or 5.0 μg/ml) or 10 different step-acetylated Flt1(1-3)-Fc samples (at 0.5, 1.0, or 5.0 μg/ml each) were passed over the Flt1(1-3)-Fc-coated chip. As shown in Figure 8, at a sub-stoichiometric ratio (0.5 μg/ml of either unmodified Flt1(1-3) or step-acetylated Flt1(1-3)-Fc vs. 0.2 μg/ml VEGF), there is not enough Flt1(1-3)-Fc (either unmodified or step-acetylated) in the solution to completely bind the VEGF. At 1.0 μg/ml, which approximates a 1:1 stoichiometric ratio, both unmodified and step-acetylated Flt1(1-3)-Fc are better able to compete for VEGF binding, but there is still insufficient Flt1(1-3)-Fc protein (either unmodified or step-acetylated) to completely bind the available VEGF. However, at 5.0 μg/ml, which is several times greater than a 1:1 stoichiometric ratio, both the Flt1(1-3)-Fc and the step-acetylated Flt1(1-3)-Fc proteins are able to bind the VEGF, regardless of the degree of acetylation. This clearly demonstrates that acetylation does not alter Flt1(1-3)-Fc's ability to bind VEGF.

(d.) Pharmacokinetic analysis of step-acetylated Flt1(1-3)-Fc

In vivo experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc and step-acetylated Flt1(1-3)-Fc protein. Balb/c mice (23-28g) were injected subcutaneously with 4mg/kg of unmodified or 10, 20, 40, 60 and 100 fold molar excess samples of step-acetylated Flt1(1-3)-Fc (3 mice for unmodified, 10, 20 and 40 fold molar excess samples and 2 mice for 60 and 100 fold molar excess samples). The mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days and 3 days after injection. The sera were assayed in an ELISA-based assay designed to detect Flt1(1-3)-Fc (described *supra*). Figure 9 details the results of this study. The Tmax for all of the Flt1(1-3)-Fc proteins tested was at the 6 hour time point but the Cmax was as follows: Unmodified Flt1(1-3)-Fc: 0.06μg/ml; 10 fold molar excess sample: - 0.7μg/ml, 20 fold molar excess sample - 2μg/ml, 40 fold molar excess sample - 4μg/ml, 60 fold molar excess sample - 2μg/ml, 100 fold molar excess sample - 1μg/ml. This results demonstrates that acetylation or pegylation of Flt1(1-3)-Fc significantly improves its pharmacokinetic profile.

Example 11: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut1: Flt1(1-3$_{\Delta B}$)-Fc.

Based on the observation that acetylated Flt1(1-3)-Fc, which has a pI

below 6, has much better pharmacokinetics than the highly positive unmodified Flt1(1-3)-Fc (pI > 9.3), it was asked whether the difference in pharmacokinetics could be attributed to the net charge of the protein, which made it stick to negatively charged extracellular matrix components, or whether there were perhaps specific locations on the surface of the Flt1(1-3)-Fc protein that constituted specific binding sites for extracellular matrix components. For example, many proteins are known to have heparin binding sites, often consisting of a cluster of basic residues. Sometimes these residues are found in a cluster on the primary sequence of the protein; some of the literature has identified "consensus sequences" for such heparin binding sites (see for example Hileman, et al., 1998, Bioessays 20(2):156-67). In other cases, the known crystal structure of a protein reveals a cluster of positively charged residues on the surface of a protein, but the residues come from different regions of the primary sequence and are only brought together when the protein folds into its tertiary structure. Thus it is difficult to deduce whether an isolated amino acid residue forms part of a cluster of basic residues on the surface of the protein. However, if there is a cluster of positively charged amino acid residues in the primary sequence, it is not unreasonable to surmise that the residues are spatially close to one another and might therefore be part of an extracellular matrix component binding site. Flt1 receptor has been studied extensively and various domains have been described (see for example Tanaka et al., 1997, Jpn. J. Cancer Res 88:867-876). Referring to the nucleic acid and amino acid sequence set forth in Figure 10A-10D

of this application, one can identify the signal sequence for secretion which is located at the beginning of the sequence and extends to the glycine coded for by nucleotides 76-78. The mature protein begins with Ser-Lys-Leu-Lys, starting at nucleotide 79 of the nucleic acid sequence. Flt1 Ig domain 1 extends from nucleotide 79 to 393, ending with the amino acids Ser-Asp-Thr. Flt1 Ig domain 2 extends from nucleotide 394 to 687 (encoding Gly-Arg-Pro to Asn-Thr-Ile), and Flt1 Ig domain 3 extends from nucleotides 688 to 996 (encoding Ile-Asp-Val to Asp-Lys-Ala). There is a bridging amino acid sequence, Gly-Pro-Gly, encoded by nucleotides 997-1005, followed by the nucleotide sequence encoding human Fc (nucleotides 1006-1701 or amino acids Glu-Pro-Lys to Pro-Gly-Lys-stop).

A more detailed analysis of the Flt1 amino acid sequence reveals that there is a cluster, namely, amino acid residues 272-281 (KNKRASVRR) of Figure 10A-10D, in which 6 out of 10 amino acid residues are basic. This sequence is located in Flt1 Ig domain 3 of the receptor (see Figure 11), which is not itself essential for binding of VEGF ligand, but which confers a higher affinity binding to ligand. An alignment of the sequence of Ig domain 3 with that of Ig domain 2 reveals that in this region, there is very poor alignment between the two Ig domains, and that there are about 10 additional amino acids in Ig domain 3. An analysis of the hydrophilicity profiles (MacVector computer software) of these two domains clearly indicates the presence of a hydrophilic region in the protein (Figure 12A-12B). These observations raised the

possibility that the actual three dimensional conformation of Flt1 Ig domain 3 allowed for some type of protrusion that is not in Flt1 Ig domain 2. To test this hypothesis, the 10 additional amino acids were deleted and the resulting protein was tested to see whether the deletion would affect the pharmacokinetics favorably without seriously compromising the affinity of the receptor for VEGF. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), is referred to as Mut1: Flt1(1-$3_{\Delta B}$)-Fc. The Mut1: Flt1(1-$3_{\Delta B}$)-Fc construct was derived from Flt1(1-3)-Fc by deletion of nucleotides 814-843 (set forth in Figure 10A-10D), which deletes the highly basic 10-amino acid residue sequence Lys-Asn-Lys-Arg-Ala-Ser-Val-Arg-Arg-Arg from Flt1 Ig domain 3.

The final DNA construct was sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut1: Flt1(1-$3_{\Delta B}$)-Fc is set forth in Figure 13A-13D.

Example 12: Construction of Flt1(1-3)-Fc basic region deletion mutant designated Mut2: Flt1(2-3 $_{\Delta B}$)-Fc.

A second deletion mutant construct, designated Mut2: Flt1(2-$3_{\Delta B}$)-Fc, was derived from the Mut1: Flt1(1-$3_{\Delta B}$)-Fc construct by deletion of Flt1 Ig domain 1 encoded by nucleotides 79-393 (see Figure 10A-10D); for convenience, nucleotides 73-78 (TCA GGT) were changed to TCC GGA. This introduced a restriction site (BspE1) without altering the associated amino acid sequence, Ser-Gly. This DNA construct, which was constructed using standard molecular biology techniques (see e.g., Molecular Cloning, A Laboratory Manual (Sambrook, et al., Cold Spring Harbor Laboratory), Current Protocols in Molecular Biology (Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY) in the mammalian expression vector pMT21 (Genetics Institute, Inc., Cambridge, MA), was also sequence-verified using an ABI 373A DNA sequencer and Taq Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA). The sequence of Mut2: Flt1(2-$3_{\Delta B}$)-Fc is set forth in Figure 14A-14C.

Example 13: Construction of Flt1(1-3)-Fc deletion mutant designated Mut3: Flt1(2-3)-Fc.

A third deletion mutate construct, designated Mut3: Flt1(2-3)-Fc, was constructed the same way as the Mut2: Flt1(2-$3_{\Delta B}$)-Fc construct, except that Flt1 Ig domain 3 was left intact (the basic region amino

acids were not deleted). The construct was constructed using standard molecular biology techniques and the final construct was sequence-verified as described *supra*. The sequence of Mut3: Flt1(2-3)-Fc is set forth in Figure 15A-15C.

Example 14: Construction of Flt(1-3)-Fc basic region N-glycosylation mutant designated Mut4: Flt1(1-3 $_{R->N}$)-Fc.

A final construct was made in which a N-glycosylation site was introduced into the middle of the basic region of Flt1 Ig domain 3. This construct was designated Mut4: Flt1(1-3$_{R->N}$)-Fc and was made by changing nucleotides 824-825 from GA to AC, consequently changing the coded Arg residue (AGA) into an Asn residue (AAC) (see Figure 10A-10D). The resulting amino acid sequence is therefore changed from Arg-Ala-Ser to Asn-Ala-Ser, which matches the canonical signal (Asn-Xxx-Ser/Thr) for the addition of a N-glycosylation site at the Asn residue. The sequence of Mut4: Flt1(1-3$_{R->N}$)-Fc is set forth in Figure 16A-16D.

Example 15: Characterization of acetylated Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, and Mut4: Flt1(1-3$_{R->N}$)-Fc mutants.

(a.) Binding to extracellular matrix components

To determine whether the three modified proteins were more or less

likely to have improved pharmacokinetic properties, Matrigel coated 96-well dishes (as described *supra*) were incubated with varying concentrations of the mutant proteins and detected with anti-human Fc/alkaline-phosphatase conjugated antibodies. As shown in Figure 18, this experiment showed that while the unmodified Flt1(1-3)-Fc protein could bind avidly to these wells, the Mut3: Flt1(2-3)-Fc protein bound somewhat more weakly, the Mut1: Flt1(1-$3_{\Delta B}$)-Fc protein bound more weakly still, and the Mut2: Flt1(2-$3_{\Delta B}$)-Fc protein showed the best profile, binding more weakly than any of the other mutant proteins. The Mut4: Flt1(1-$3_{R->N}$)-Fc glycosylation mutant protein showed only marginal benefit on the Matrigel assay. These results confirm the hypothesis that a linear sequence of positive amino acids can be deleted from the primary sequence resulting in a decrease in charge interaction with extracellular matrix components.

(b.) Binding of Mut1: Flt1(1-$3_{\Delta B}$)-Fc and Mut4: Flt1(1-$3_{R->N}$)-Fc in a Biacore-based assay.

Unmodified and acetylated Flt1(1-3)-Fc and genetically modified Mut1: Flt1(1-$3_{\Delta B}$)-Fc and Mut4: Flt1(1-$3_{R->N}$)-Fc proteins where tested in a Biacore-based assay to evaluate their ability to bind to the Flt1 ligand, VEGF. In this assay, unmodified Flt1(1-3)-Fc protein (0.25, 0.5, or 1.0 μg/ml) was immobilized on the surface of a Biacore chip (see Biacore Instruction Manual, Pharmacia, Inc., Piscataway, NJ, for standard procedures) and a solution containing 0.1 μg/ml VEGF and either

purified or COS cell supernatant containing unmodified Flt1(1-3)-Fc (at approximately (0.25, 0.5, or 1.0 μg/ml), purified acetylated Flt1(1-3)-Fc (at (0.25, 0.5, or 1.0 μg/ml), COS cell supernatant containing Mut1: Flt1(1-$3_{\Delta B}$)-Fc (at approximately (0.25, 0.5, or 1.0 μg/ml), or COS cell supernatant containing Mut4: Flt1(1-$3_{R->N}$)-Fc (at approximately (0.25, 0.5, or 1.0 μg/ml) were passed over the Flt1(1-3)-Fc-coated chip. As shown in Figure 17, at the sub-stoichiometric ratio (0.25 μg/ml Flt1(1-3)-Fc of unmodified, acetylated or genetically modified samples vs. 01. μg/ml VEGF), there is insufficient Flt1(1-3)-Fc protein to block binding of VEGF to the Flt1(1-3)-Fc immobilized on the Biacore chip. At 0.5 μg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, the stoichiometric ratio approximates 1:1 and there is an increased ability to block VEGF binding to the Biacore chip. At 1.0 μg/ml of unmodified, acetylated or genetically modified Flt1(1-3)-Fc proteins, which is approximately a 10:1 stoichiometric ratio, the Flt1(1-3)-Fc proteins are able to block binding of VEGF to the Biacore chip, but they are not equivalent. Unmodified, acetylated, and Mut1: Flt1(1-$3_{\Delta B}$)-Fc are essentially equal in their ability to block VEGF binding, whereas Mut4: Flt1(1-$3_{R->N}$)-Fc is somewhat less efficient at blocking binding. These results confirm the hypothesis that it is possible to reduce the non-specific binding of a positively charged molecule by genetically removing a linear sequence of predominantly negatively charged amino acids.

(c.) Binding of Mut1: Flt1(1-3$_{\Delta B}$)-Fc, Mut2: Flt1(2-3$_{\Delta B}$)-Fc, Mut3: Flt1(2-3)-Fc, and in an ELISA-based assay.

To determine whether the three mutant proteins could bind the Flt1 ligand VEGF, binding experiments were done in which 96-well plates coated with VEGF were incubated with varying concentrations of the respective mutant protein, and after washing, the amount bound was detected by incubating with an alkaline phosphatase conjugated anti-human Fc antibody and quantitated colorimetrically by the addition of an appropriate alkaline phosphatase substrate. As shown in Figure 19, this experiment showed that all the mutant proteins could bind VEGF similarly, at the concentrations tested.

Example 16: Pharmacokinetic analysis of acetylated Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, and unmodified Flt1(1-3)-Fc.

In vivo experiments were designed to assess the pharmacokinetic profiles of unmodified Flt1(1-3)-Fc, Mut1: Flt1(1-3$_{\Delta B}$)-Fc, and 40 fold molar excess acetylated Flt1(1-3)-Fc protein. Balb/c mice (25-30g) were injected subcutaneously with 4mg/kg of unmodified Flt1(1-3)-Fc, 40 fold molar excess acetylated Flt1(1-3)-Fc, and Mut1: Flt1(1-3$_{\Delta B}$)-Fc proteins (4 mice each). These mice were tail bled at 1, 2, 4, 6, 24 hours, 2 days, 3 days, and 5 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc protein which involves coating an ELISA plate with VEGF, binding the Flt1(1-3)-Fc and

reporting with an anti-Fc antibody linked to alkaline phosphatase. As shown in Figure 20, the Cmax for these reagents was as follows: Unmodified Flt1(1-3)-Fc - 0.15μg/ml; 40 fold molar excess acetylated Flt1(1-3)-Fc - 1.5μg/ml; and Mut1: Flt1(1-$3_{\Delta B}$)-Fc - 0.7μg/ml.

Example 17: Modified Flt1 receptor vector construction

The rationale for constructing modified versions of the Flt1 receptor (also known as VEGFR1) was based on the observation that the protein sequence of Flt1 was highly basic, and was therefore likely to stick to extracellular matrix (ECM). The highly basic nature of Flt1 probably explains why unmodified Flt1(1-3)-Fc (described *supra*) has poor pharmacokinetics that make it difficult to use as a therapeutic agent. As described *supra*, the chemically modified form of 40 fold molar excess acetylated Flt1(1-3)-Fc, hereinafter termed A40, exhibited a greatly improved pharmacokinetic (PK) profile over the non-acetylated Flt1(1-3)-Fc. Therefore, attempts were made to engineer DNA molecules that could be used to recombinantly express modified forms of a Flt1 receptor molecule that would possess the improved PK profile exhibited by A40 and still maintain the ability to bind tightly to VEGF.

It is known in the literature that the first Ig domain of Flt1 (which has a net charge of +5 at neutral pH) is not essential for tight binding to VEGF, so this domain was deleted. The third Ig domain (having a net charge of +11) is not essential for binding, but confers higher affinity

for VEGF than the second Ig domain, so instead of deleting it entirely, it was replaced with the equivalent domains of the Flt1 receptor relatives Flk1 (also known as VEGFR2) and Flt4 (also known as VEGFR3). These chimeric molecules (denoted R1R2 (Flt1.D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a) and R1R3 (Flt1D2.VEGFR3D3-FcΔC1(a) and VEGFR1R3-FcΔC1(a) respectively, wherein R1 and Flt1D2 = Ig domain 2 of Flt1 (VEGFR1); R2 and Flk1D3 = Ig domain 3 of Flk1 (VEGFR2); and R3 and VEGFR3D3 = Ig domain 3 of Flt4 (VEGFR3)) were much less sticky to ECM, as judged by an *in vitro* ECM binding assay as described *infra* , had greatly improved PK as described *infra*. In addition, these molecules were able to bind VEGF tightly as described *infra* and block phosphorylation of the native Flk1 receptor expressed in endothelial cells as described *infra*.

(a) Construction of the expression plasmid pFlt1D2.Flk1D3.FcΔC1(a)

Expression plasmids pMT21.Flt1(1-3).Fc (6519bp) and pMT21.Flk-1(1-3).Fc (5230bp) are plasmids that encode ampicillin resistance and Fc-tagged versions of Ig domains 1-3 of human Flt1 and human Flk1, respectively. These plasmids were used to construct a DNA fragment consisting of a fusion of Ig domain 2 of Flt1 with Ig domain 3 of Flk1, using PCR amplification of the respective Ig domains followed by further rounds of PCR to achieve fusion of the two domains into a

single fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')

3': Flt1D2-Flk1D3.as (5'-CGGACTCAGAACCACATCTATGATTGTATTGGT-3')

The 5' amplification primer encodes a BspE1 restriction enzyme site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (corresponding to amino acids 27-33 of Figure 21A-21C). The 3' primer encodes the reverse complement of the 3' end of Flt1 Ig domain 2 fused directly to the 5' beginning of Flk1 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of Figure 21A-21C) and continuing into VVLS (corresponding to amino acids 127-130 of Figure 21A-21C) of Flk1.

For Ig domain 3 of Flk1, the 5' and 3' amplification primers were as follows:

5': Flt1D2-Flk1D3.s (5'-ACAATCATAGATGTGGTTCTGAGTCCGTCTCATG G-3')

3': Flk1D3/apa/srf.as (5'-GATAATGCCCGGGCCCTTTTCATGGACCCTGAC AAATG-3')

The 5' amplification primer encodes the end of Flt1 Ig domain 2 fused directly to the beginning of Flk1 Ig domain 3, as described above. The 3' amplification primer encodes the end of Flk1 Ig domain 3, defined by the amino acids VRVHEK (corresponding to amino acids 223-228 of Figure 21A-21C), followed by a bridging sequence that includes a recognition sequence for the restriction enzyme Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 229-231 of Figure 21A-21C.

After a round of PCR amplification to produce the individual domains, the products were combined in a tube and subjected to a further round of PCR with the primers bsp/flt1D2 and Flk1D3/apa/srf.as (described *supra*) to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 614bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/ΔB2.Fc, to create the plasmid pMT21/Flt1D2.Flk1D3.Fc. The nucleotide sequence of the Flt1D2-Flk1D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 702bp fragment was transferred into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)B2-FcΔC1(a) to produce the plasmid pFlt1D2.Flk1D3.FcΔC1(a). The complete DNA and deduced amino acid sequences of the Flt1D2.Flk1D3.FcΔC1(a) chimeric molecule is set forth in Figure 21A-21C.

(b) Construction of the expression plasmid pFlt1D2VEGFR3D3FcΔC1(a)

The expression plasmid pMT21.Flt1(1-3).Fc (6519bp) encodes ampicillin resistance and an Fc-tagged version of Ig domains 1-3 of human Flt1 receptor. This plasmid was used to produce a DNA fragment containing Ig domain 2 of Flt1 by PCR. RNA from the cell line HEL921.7 was used to produce Ig domain 3 of Flk1, using standard RT-PCR methodology. A further round of PCR amplification was used to achieve fusion of the two Ig domains into a single fused fragment. For Ig domain 2 of Flt1, the 5' and 3' amplification primers were as follows:

5': bsp/flt1D2 (5'-GACTAGCAGTCCGGAGGTAGACCTTTCGTAGAGATG-3')

3': Flt1D2.VEGFR3D3.as(TTCCTGGGCAACAGCTGGATATCTATGATTGTA TTGGT)

The 5' amplification primer encodes a BspE1 restriction site upstream of Ig domain 2 of Flt1, defined by the amino acid sequence GRPFVEM (corresponding to amino acids 27-33 of Figure 22A-22C). The 3' amplification primer encodes the reverse complement of the end of Flt1 Ig domain 2 fused directly to the beginning of VEGFR3 Ig domain 3, with the fusion point defined as TIID of Flt1 (corresponding to amino acids 123-126 of Figure 22A-22C) and continuing into IQLL of VEGFR3 (corresponding to amino acids 127-130 of Figure 22A-22C).

For Ig domain 3 of VEGFR3, the 5' and 3' primers used for RT-PCR were as follows:

5' R3D3.s (ATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGCTGGTA)

3': R3D3.as (ATTTTCATGCACAATGACCTCGGTGCTCTCCCGAAATCG)

Both the 5' and 3' amplification primers match the sequence of VEGFR3. The 296bp amplification product of this RT-PCR reaction was isolated by standard techniques and subjected to a second round of PCR to add suitable sequences to allow for fusion of the Flt1D2 with the Flk1D3 domains and fusion of the Flk1D3 and Fc domains via a GPG bridge (see below). The amplification primers were as follows:

5':Flt1D2.VEGFR3D3.s
(TCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAG)

3': VEGFR3D3/srf.as
(GATAATGCCCGGGCCATTTTCATGCACAATGACCTCGGT)

The 5' amplification primer encodes the 3' end of Flt1 Ig domain 2 fused directly to the beginning (5' end) of VEGFR3 Ig domain 3, as described above. The 3' amplification primer encodes the 3' end of VEGFR3 Ig domain 3, defined by the amino acids VIVHEN (corresponding

to amino acids 221-226 of Figure 22A-22C), followed by a bridging sequence that includes a recognition sequence for Srf1, and encodes the amino acids GPG. The bridging sequence corresponds to amino acids 227-229 of Figure 22A-22C.

After one round (for Flt1 Ig domain 2) or two rounds (for Flt4 Ig domain 3) of PCR to produce the individual Ig domains, the PCR products were combined in a tube and subjected to a further round of PCR amplification with the amplification primers bsp/flt1D2 and VEGFR3D3/srf.as described *supra*, to produce the fusion product. This PCR product was subsequently digested with the restriction enzymes BspEI and SmaI and the resulting 625bp fragment was subcloned into the BspEI to SrfI restriction sites of the vector pMT21/Flt1ΔB2.Fc (described *supra*), to create the plasmid pMT21/Flt1D2.VEGFR3D3.Fc. The sequence of the Flt1D2-VEGFR3D3 gene fusion insert was verified by standard sequence analysis. This plasmid was then digested with the restriction enzymes EcoRI and SrfI and the resulting 693bp fragment was subcloned into the EcoRI to SrfI restriction sites of the plasmid pFlt1(1-3)ΔB2-FcΔC1(a) to produce the plasmid designated pFlt1D2.VEGFR3D3.FcΔC1(a). The complete DNA deduced amino acid sequence of the Flt1D2.VEGFR3D3.FcΔC1(a) chimeric molecule is set forth in Figure 22A-22C.

Example 18: Extracellular Matrix Binding (ECM) Binding Assay

ECM-coated plates (Becton Dickinson catalog # 35-4607) were rehydrated with warm DME supplemented with glutamine (2mM), 100U penicillin, 100U streptomycin, and 10% BCS for at least 1 hr. before adding samples. The plates were then incubated for 1 hr. at room temperature with varying concentrations of Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) starting at 10nM with subsequent 2-fold dilutions in PBS plus 10% BCS. The plates were then washed 3 times with PBS plus 0.1% Triton-X and incubated with alkaline phosphatase-conjugated anti-human Fc antibody (Promega, 1:4000 in PBS plus 10% BCS) for 1 hr. at room temperature. The plates were then washed 4 times with PBS 0.1% Triton-X and alkaline phosphatase buffer/pNPP solution (Sigma) was added for color development. Plates were read at l = 405-570nm. The results of this experiment are shown in Figure 23 and demonstrate that the Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) proteins are considerably less sticky to the ECM as compared to the Flt1(1-3)-Fc protein.

Example 19: Transient expression of pFlt1D2.Flk1D3.FcΔC1(a) in CHO-K1 (E1A) cells

A large scale (2L) culture of E. coli DH10B cells carrying the pFlt1D2.Flk1D3.FcΔC1(a) plasmid described *supra* in Example 17(a) was grown overnight in Terrific Broth (TB) plus 100μg/ml ampicillin. The

next day, the plasmid DNA was extracted using a QIAgen Endofree Megaprep kit following the manufacturer's protocol. The concentration of the purified plasmid DNA was determined by standard techniques using a UV spectrophotometer and fluorometer. The plasmid DNA was verified by standard restriction enzyme digestion of aliquots using the restriction enzymes EcoRI plus NotI and AseI. All restriction enzyme digest fragments corresponded to the predicted sizes when analyzed on a 1% agarose gel.

Forty 15 cm petri plates were seeded with CHO-K1/E1A cells at a density of 4 x 106 cells/plate. Plating media was Gibco Ham's F-12 supplemented with 10% Hyclone Fetal Bovine Serum (FBS), 100U penicillin/100U streptomycin and glutamine (2mM). The following day each plate of cells was transfected with 6 μg of the pFlt1D2.Flk1D3.FcΔC1(a) plasmid DNA using Gibco Optimem and Gibco Lipofectamine in 12 ml volume, following the manufacturer's protocol. Four hours after adding the transfection mix to the cells, 12 ml/plate of Optimem supplemented with 10% FBS was added. Plates were incubated at 37°C in a 5% CO_2 incubator overnight. The following day the media was removed from each plate and 25 ml expression media (Gibco CHO-S-SFM II supplemented with glutamine (2mM) and 1mM sodium butyrate) was added. The plates were incubated at 37°C for 3 days. After 3 days of incubation, the media was aspirated from each plate and centrifuged at 400 rpm in a swinging bucket rotor to pellet

cells. The supernatant was decanted into sterile 1L bottles and purification of the expressed protein was performed as described *infra*.

Example 20: Construction pVEGFR1R2-FcΔC1(a) expression vector

The pVEGFR1R2.FcΔC1(a) expression plasmid was constructed by insertion of DNA encoding amino acids SDT (corresponding to amino acids 27-29 of Figure 24A-24C) between Flt1d2-Flk1d3-FcΔC1(a) amino acids 26 and 27 of Figure 21A-21C (GG) and removal of DNA encoding amino acids GPG corresponding to amino acids 229-231 of Figure. The SDT amino acid sequence is native to the Flt1 receptor and was added back in to decrease the likelihood of heterogeneous N-terminal processing. The GPG (bridging sequence) was removed so that the Flt1 and Flk1 Ig domains were fused directly to one another. The complete DNA and deduced amino acid sequences of the pVEGFR1R2.FcΔC1(a) chimeric molecule is set forth in Figure 24A-24C.

Example 21: Cell Culture Process Used to Produce Modified Flt1 Receptors

(a) Cell Culture Process Used to Produce Flt1D2.Flk1D3.FcΔC1(a)

The process for production of Flt1D2.Flk1D3.FcΔC1(a) protein using the

expression plasmid pFlt1D2.Flk1D3.FcΔC1(a) described *supra* in Example 1 involves suspension culture of recombinant Chinese hamster ovary (CHO K1/E1A) cells which constitutively express the protein product. The cells are grown in bioreactors and the protein product is isolated and purified by affinity and size exclusion chromatography. The process is provided in greater detail below.

Cell Expansion

Two confluent T-225 cm^2 flasks containing the Flt1D2.Flk1D3.FcΔC1(a) expressing cell line were expanded by passaging cells into eight T-225 cm^2 flasks in medium (GMEM + 10% serum, GIBCO) and incubated at 37°C and 5% CO_2. When the flasks approached confluence (approximately 3 to 4 days) the cells were detached using trypsin. Fresh medium was added to protect the cells from further exposure to the trypsin. The cells were centrifuged and resuspended in fresh medium then transferred to eight 850 cm^2 roller bottles and incubated at 37°C and 5% CO_2 until confluent.

Suspension Culture in Bioreactors

Cells grown in roller bottles were trypsinized to detach them from the surface and washed with suspension culture medium. The cells are aseptically transferred to a 5L bioreactor (New Brunswick Celligen Plus) where the cells are grown in 3.5L of suspension culture. The

suspension culture medium was a glutamine-free low glucose modification of IS-CHO (Irvine Scientific) to which 5% fetal bovine serum (Hyclone), GS supplement (Life Technologies) and 25 μM methionine sulfoximine (Sigma) was added. The pH was controlled at 7.2 by addition of carbon dioxide to the inlet gas or by addition of a liquid solution of sodium carbonate to the bioreactor. Dissolved oxygen level was maintained at 30% of saturation by addition of oxygen or nitrogen to the inlet gas and temperature controlled at 37°C. When a density of 4 $x10^6$ cells/mL was reached the cells were transferred to a 40L bioreactor containing the same medium and setpoints for controlling the bioreactor. The temperature setpoint was reduced to 34°C to slow cell growth and increase the relative rate of protein expression.

(b) Cell Culture Process Used to Produce Flt1D2.VEGFR3D3.FcΔC1(a)

The same methodologies as described *supra* for Flt1D2.Flk1D3.FcΔC1(a) were used to produce Flt1D2.VEGFR3D3.FcΔC1(a).

Example 22: Harvest and Purification of Modified Flt1 Receptors

(a) Harvest and Purification of Flt1D2.Flk1D3.FcΔC1(a)

The product protein was aseptically harvested from the bioreactor while retaining cells using Millipore Prostak tangential-flow filtration modules and a low-shear mechanical pump (Fristam). Fresh medium was added to the bioreactor to replace that removed during the harvest filtration. Approximately 40L of harvest filtrate was then loaded onto a 400 mL column containing Protein A Sepharose resin (Amersham Pharmacia). After loading the resin was washed with buffer containing 10 mM sodium phosphate, 500 mM sodium chloride, pH 7.2 to remove any unbound contaminating proteins. Flt1D2.Flk1D3.FcΔC1(a) protein was eluted with a pH 3.0 citrate buffer. The eluted protein was neutralized by addition of Tris base and frozen at -20°C.

Several frozen lots of Flt1D2.Flk1D3.FcΔC1(a) protein from the Protein A step above were thawed, pooled and concentrated using a Millipore 30kD nominal molecular weight cutoff (NMWCO) tangential flow filtration membrane. The protein was transferred to a stirred cell concentrator (Millipore) and further concentrated to 30 mg/mL using a 30kD NMWCO membrane. The concentrated protein was loaded onto a size exclusion column packed with Superdex 200 resin (Amersham Pharmacia) that was equilibrated with phosphate buffered saline plus 5% glycerol. The same buffer was used to run the column. The fractions corresponding to Flt1D2.Flk1D3.FcΔC1(a) dimer were pooled, sterile filtered through a 0.22 micron filter, aliquoted and frozen.

(b) Harvest and Purification of Flt1D2.VEGFR3D3.FcΔC1(a)

The same methodologies as described *supra* for Flt1D2.Flk1D3.FcΔC1(a) were used to harvest and purify Flt1D2.VEGFR3D3.FcΔC1(a).

Example 23: Phosphorylation Assay for Transiently Expressed VEGFR2

Primary human umbilical vein endothelial cells (HUVECs), passage 4-6, were starved for 2 hrs in serum-free DME high glucose media. Samples containing 40 ng/ml (1nM) human VEGF165, which is a ligand for the VEGF receptors Flt1, Flk1 and Flt4(VEGFR3) were prepared and were preincubated for 1 hr. at room temperature with varying amounts of the modified Flt1 receptors Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), Flt1D2Flk1D3.FcΔC1(a) and Flt1D2VEGFR3D3.FcΔC1(a) in serum-free DME-high glucose media containing 0.1% BSA. Cells were challenged for 5 minutes with the samples prepared above +/- VEGF165, followed by whole cell lysis using complete lysis buffer. Cell lysates were immunoprecipitated with an antibody directed against the C-terminus of VEGFR2 receptor. The immunoprecipitated lysates were loaded onto 4-12% SDS-PAGE Novex gel and then transferred to PVDF membrane using standard transfer methodologies. Detection of phosphorylated VEGFR2 was done by immunoblotting with the anti-phospho Tyrosine mAb called 4G10 (UBI) and developed using ECL-reagent (Amersham).

Figures 25A-25C and 26A-26B show the results of this experiment. Figure 25A-25C reveals that detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are phosphorylated to varying levels depending on which modified Flt1 receptor is used during the preincubations with VEGF. As is seen in Figure 25A, at a 1.5 molar excess of either Flt1(1-3)-Fc , Flt1(1-3)-Fc (A40) or transient Flt1D2Flk1D3.FcΔC1(a) there is complete blockage of receptor stimulation by these three modified Flt1 receptors as compared to control media challenge. In contrast, transient Flt1D2VEGFR3D3.FcΔC1(a) does not show significant blockage at this molar excess, as compared with VEGF positive control challenge. Similar results are seen in Figure 25B, where the modified Flt receptors are in a 3-fold molar excess to VEGF165 ligand. In Figure 25C, where the modified Flt1 receptors are in a 6-fold molar excess to VEGF165 ligand, transient Flt1D2VEGFR3D3.FcΔC1(a) can now be shown to be partially blocking VEGF165-induced stimulation of cell-surface receptors.

In Figure 26A-26B, detection by Western blot of tyrosine phosphorylated VEGFR2(Flk1) by VEGF165 ligand stimulation shows that cell-surface receptors are not phosphorylated by challenge samples which have VEGF165 preincubated with 1 and 2 fold molar excess (Figure 26A) or 3 and 4 fold molar excess (Figure 26B) of either transient Flt1D2Flk1D3.FcΔC1(a), stable Flt1D2Flk1D3.FcΔC1(a), or transient VEGFR1R2-FcΔC1(a). At all modified Flt1 receptor

concentrations tested there is complete binding of VEGF165 ligand during the preincubation, resulting in no detectable stimulation of cell-surface receptors by unbound VEGF165 as compared to control media challenge.

Example 24: Cell Proliferation Bioassay

The test cell population is MG87 cells that have been stably transfected with a expression plasmid that contains a DNA insert encoding the VEGFR2(Flk1) extracellular domain fused to the TrkB intracellular kinase domain, thus producing a chimeric molecule. The reason the TrkB intracellular kinase domain was used rather than the native VEGFR2(Flk1) intracellular kinase domain is that the intracellular kinase domain of VEGFR2(Flk1) does not cause a strong proliferative response when stimulated by VEGF165 in these cells. It is known that MG87 cells containing full length TrkB receptor give a robust proliferative response when stimulated with BDNF, so the TrkB intracellular kinase domain was engineered to replace the intracellular kinase domain of VEGFR2(Flk1) to take advantage of this proliferative response capability.

5 x 10^3 cells/well were plated in a 96 well plate and allowed to settle for 2 hrs at 37°C. The following modified Flt receptors Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a), plus an irrelevant receptor termed Tie2-Fc as a negative control, were titrated

from 40nM to 20pM and incubated on the cells for 1hr at 37°C. Human recombinant VEGF165 in defined media was then added to all the wells at a concentration of 1.56nM. The plates were incubated for 72 hrs at 37°C and then MTS (Owen's reagent, Promega) added and the plates were incubated for an additional for 4 hrs. Finally, the plates were read on a spectrophotometer at 450/570nm. The results of this experiment are shown in Figure 27. The control receptor Tie2-Fc does not block VEGF165-induced cell proliferation at any concentration whereas Flt1D2.Flk1D3.FcΔC1(a) blocks 1.56nM VEGF165 with a half maximal dose of 0.8nM. Flt1(1-3)-Fc and Flt1D2.VEGFR3D3.FcΔC1(a) are less effective in blocking VEGF165 in this assay with a half maximal dose of ~ 2nM. VEGF165 alone gives a reading of 1.2 absorbance units and the background is 0.38 absorbance units.

Example 25: Binding Stoichiometry of Modified Flt Receptors to VEGF165

(a) BIAcore Analysis

The stoichiometry of Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a) interaction with human VEGF165 was determined by measuring either the level of VEGF saturation binding to the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) surfaces or measuring concentration of VEGF165 needed to completely prevent binding of Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) to VEGF BIAcore chip surface.

Modified Flt receptors Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a), were captured with an anti-Fc specific antibody that was first immobilized on a Biacore chip (BIACORE) using amine-coupling chemistry. A blank antibody surface was used as a negative control. VEGF165 was injected at a concentration of 1 nM, 10 nM, and 50 nM over the Flt1D2Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a) surfaces at 10 μl/min for one hour. A real-time binding signal was recorded and saturation binding was achieved at the end of each injection. Binding stoichiometry was calculated as a molar ratio of bound VEGF165 to the immobilized Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a), using the conversion factor of 1000 RU equivalent to 1 ng/ml. The results indicated binding stoichiometry of one VEGF165 dimeric molecule per one Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) molecule (Figure 28).

In solution, Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) at a concentration of 1nM (estimated to be 1000 times higher than the KD of the Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a)/VEGF165 interaction) were mixed with varied concentrations of VEGF165. After one hour incubation, concentrations of the free Flt1D2Flk1D3.FcΔC1(a) in solution were measured as a binding signal to an amine-coupled VEGF165 surface. A calibration curve was used to convert the Flt1D2Flk1D3.FcΔC1(a) BIAcore binding signal to its molar concentration. The data showed that the addition of 1 nM VEGF165 into

the Flt1D2Flk1D3.FcΔC1(a) solution completely blocked Flt1D2Flk1D3.FcΔC1(a) binding to the VEGF165 surface. This result suggested the binding stoichiometry of one VEGF165 molecule per one Flt1D2Flk1D3.FcΔC1(a) molecule (Figure 29 and Figure 30). When the concentration of Flt1D2Flk1D3.FcΔC1(a) was plotted as a function of added concentration of VEGF165, the slope of the linear portion was -1.06 for Flt1D2Flk1D3.FcΔC1(a) and -1,07 for VEGFR1R2-FcΔC1(a). The magnitude of the slope, very close to negative one, was indicative that one molecule of VEGF165 bound to one molecule of either Flt1D2Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a).

(b) Size Exclusion Chromatography

Flt1D2Flk1D3.FcΔC1(a) was mixed with a 3-fold excess of VEGF165 and the receptor-ligand complex was purified using a Pharmacia Superose 6 size exclusion chromatography column. The receptor-ligand complex was then incubated in a buffer containing 6M guanidine hydrochloride in order to dissociate it into its component proteins. Flt1D2Flk1D3.FcΔC1(a) was separated from VEGF165 using Superose 6 size exclusion chromatography column run in 6M guanidium chloride. In order to determine complex stoichiometry, several injections of Flt1D2Flk1D3.FcΔC1(a) and VEGF165 were made and peak height or peak integrated intensity was plotted as a function of the concentration of injected protein. The calibration was done under condition identical to one used in separating components of Flt1D2Flk1D3.FcΔC1(a)/VEGF

complex. Quantification of the Flt1D2Flk1D3.FcΔC1(a)/VEGF complex composition was based on the calibration curves. The results of this experiment are set forth in Figure 28, which shows the ratio of VEGF165 to Flt1D2Flk1D3.FcΔC1(a) in a complex to be 1:1.

Example 26: Determination of the Binding Stoichiometry of Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex by Size Exclusion Chromatography

Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex Preparation

VEGF165 (concentration = 3.61 mg/ml) was mixed with CHO cell transiently expressed Flt1D2.Flk1D3.FcΔC1(a) (concentration = 0.9 mg/ml) in molar ratio of 3:1 (VEGF165:Flt1D2.Flk1D3.FcΔC1(a)) and incubated overnight at 4°C.

(a) Size Exclusion Chromatography (SEC) under native conditions

To separate the complex from excess of unbound VEGF165, 50 µl of the complex was loaded on a Pharmacia Superose 12 PC 3.2/30 which was equilibrated in PBS buffer. The sample was eluted with the same buffer at flow rate 40µl/min. at room temperature. The results of this SEC are shown in Figure 31. Peak #1 represents the complex and peak #2 represents unbound VEGF165. Fractions eluted between 1.1 and 1.2

ml were combined and guanidinium hydrochloride (GuHCl)was added to a final concentration 4.5M to dissociate the complex.

(b) Size Exclusion Chromatography (SEC) under dissociative conditions

To separate the components of the receptor-ligand complex and to determine their molar ratio, 50µl of dissociated complex as described *supra* was loaded onto a Superose 12 PC 3.2/30 equilibrated in 6M GuHCl and eluted with the same solution at a flow rate 40µl/min. at room temperature. The results of this SEC are shown in Figure 32. Peak #1 represents Flt1D2Flk1D3.FcΔC1(a) and peak #2 represents VEGF165.

(c) Calculation of Flt1D2Flk1D3.FcΔC1(a):VEGF165 Complex Stoichiometry

The stoichiometry of the receptor-ligand complex was determined from the peak area or the peak height of the components. Concentrations of VEGF165 and Flt1D2Flk1D3.FcΔC1(a) corresponding to the peak height or peak area, respectively, were obtained from the standard curves for VEGF165 and Flt1D2Flk1D3.FcΔC1(a). To obtain a standard curve, four different concentrations (0.04 mg/ml -0.3mg/ml) of either component were injected onto a Pharmacia Superose 12 PC 3.2/30 column equilibrated in 6M guanidinium chloride and eluted with the same

solution at flow rate 40µl/min. at room temperature. The standard curve was obtained by plotting peak area or peak height vs protein concentration. The molar ratio of VEGF165:Flt1D2Flk1D3.FcΔC1(a) determined from the peak area of the components was 1.16. The molar ratio of VEGF165:Flt1D2Flk1D3.FcΔC1(a) determined from the peak height of the components was 1.10.

Example 27: Determination of the Stoichiometry of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 Complex by Size Exclusion Chromatography with On-Line Light Scattering

Complex preparation

VEGF165 was mixed with CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a) protein in molar ratio of 3:1 (VEGF165:Flt1D2Flk1D3.FcΔC1(a)) and incubated overnight at 4°C.

(a) Size Exclusion Chromatography (SEC) with On-Line Light Scattering

Size exclusion chromatography column with a MiniDawn on-line light scattering detector (Wyatt Technology, Santa Barbara, California) and refractive index (RI) detectors (Shimadzu, Kyoto, Japan) was used to determine the molecular weight (MW) of the receptor-ligand complex. Samples were injected onto a Superose 12 HR 10/30 column

(Pharmacia) equilibrated in PBS buffer and eluted with the same buffer at flow rate 0.5 ml/min. at room temperature. As shown in Figure 33, the elution profile shows two peaks. Peak #1 represents the receptor-ligand complex and peak #2 represents the unbound VEGF165. MW was calculated from LS and RI signals. The same procedure was used to determine MW of the individual components of the receptor-ligand complex. The results of these determinations are as follows: MW of the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex at the peak position is 157 300 (Figure 33), the MW of VEGF165 at the peak position is 44 390 (Figure 34) and the MW of R1R2 at the peak is 113 300 (Figure 35).

These data indicated that the stoichiometry of the Flt1D2Flk1D3.FcΔC1(a)/VEGF complex is 1:1 as its corresponds to the sum of molecular weights for Flt1D2Flk1D3.FcΔC1(a) and VEGF165. Importantly, this method conclusively proved that the Flt1D2Flk1D3.FcΔC1(a)/VEGF165 complex was indeed composed of only one molecule of VEGF165 ligand and only one molecule of the Flt1D2Flk1D3.FcΔC1(a).

Example 28: Peptide Mapping of Flt1D2.Flk1D3.FcΔC1(a)

The disulfide structures and glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a) were determined by a peptide mapping method. In this method, the protein was first cleaved with trypsin. Tryptic fragments were analyzed and identified by HPLC coupled with mass

spectrometry, in addition to an N-terminal sequencing technique. Reduction of the tryptic digest was employed to help identify disulfide-bond-containing fragments. Treatment of the tryptic digest with PNGase F (Glyko, Novato, CA) was employed to help identify fragments with N-linked glycosylation sites. The results are summarized in the accompanying Figure 36.

There are a total of ten cysteines in Flt1D2.Flk1D3.FcΔC1(a); six of them belong to the Fc region. Cys27 has been confirmed to be disulfide bonded to Cys76. Cys121 is confirmed to be disulfide bonded to Cys 182. The first two cysteines in the Fc region (Cys211 and Cys214) form an intermolecular disulfide bond with the same two cysteines in another Fc chain. However, because these two cysteines can not be separated enzymatically from each other, it can not be determined whether disulfide bonding is occurring between same cysteines (Cys211 to Cys211, for example) or between Cys211 and Cys214. Cys216 is confirmed to be disulfide bonded to Cys306. Cys 352 is confirmed to be disulfide bonded to Cys410.

There are five possible N-linked glycosylation sites in Flt1D2.Flk1D3.FcΔC1(a). All five of them are found to be glycosylated to varying degrees. Complete glycosylation was observed at Asn33 (amino acid sequence NIT), Asn193 (amino acid sequence NST), and Asn282 (amino acid sequence NST). In addition, partial glycosylation is

observed on Asn65 and Asn120. Sites of glycosylation are highlighted by underline in the Figure 36.

Example 29: Pharmacokinetic Analysis of Modified Flt Receptors

(a) Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)

Balb/c mice (25-30g) were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a), CHO stably expressed Flt1D2.Flk1D3.FcΔC1(a), and CHO transiently expressed VEGFR1R2-FcΔC1(a). The mice were tail bled at 1, 2, 4, 6, 24hrs, 2 days, 3 days and 6 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a). The ELISA involves coating an ELISA plate with VEGF165, binding the detect Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or VEGFR1R2-FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. The results of this experiments are shown in Figure 37. The T_{max} for Flt1(1-3)-Fc (A40) was at 6 hrs while the T_{max} for the transient and stable Flt1D2.Flk1D3.FcΔC1(a) and the transient VEGFR1R2-FcΔC1(a) was 24hrs. The C_{max} for Flt1(1-3)-Fc (A40) was 8μg/ml. For both transients (Flt1D2.Flk1D3.FcΔC1(a) and VEGFR1R2-FcΔC1(a)) the C_{max}

was 18μg/ml and the C_{max} for the stable VEGFR1R2-FcΔC1(a) was 30μg/ml.

(b) Pharmacokinetic analysis of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a)

Balb/c mice (25-30g) were injected subcutaneously with 4mg/kg of Flt1(1-3)-Fc (A40), CHO transiently expressed Flt1D2.Flk1D3.FcΔC1(a) and CHO transiently expressed Flt1D2.VEGFR3D3.FcΔC1(a). The mice were tail bled at 1, 2, 5, 6, 7, 8, 12, 15 and 20 days after injection. The sera were assayed in an ELISA designed to detect Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). The ELISA involves coating an ELISA plate with 165, binding the Flt1(1-3)-Fc, Flt1D2.Flk1D3.FcΔC1(a) or Flt1D2.VEGFR3D3.FcΔC1(a) and reporting with an anti-Fc antibody linked to horse radish peroxidase. Flt1(1-3)-Fc (A40) could no longer be detected in the serum after day 5 whereas , Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) were detectable for 15 days or more. The results of this experiment are shown in Figure 38.

Example 30: Evaluation of the Ability of Flt1D2.Flk1D3.FcΔC1(a) to Inhibit Tumor Growth In Vivo

To evaluate the ability of Flt1D2.Flk1D3.FcΔC1(a) to inhibit tumor growth in vivo a model in which tumor cell suspensions are implanted

subcutaneously on the right flank of male severe combined immunodeficiency (SCID) mice was employed. Two cell lines, the human HT-1080 fibrosarcoma cell line (ATCC accession no. CCL-121) and the rat C6 glioma cell line (ATCC accession no. CCL-107), each of which exhibit distinctly different morphologies and growth characteristics, were used in the assay. The first dose of Flt1D2.Flk1D3.FcΔC1(a) (at 25mg/Kg or as indicated in Figures 39 and 40) was given on the day of tumor implantation. Animals subsequently received subcutaneous injections of Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) or vehicle either every other day (EOD) or two times per week (2X/wk) for a period of 2 weeks. After 2 weeks, animals were perfused with fixative, tumors were removed and samples were blinded. Tumor volume was determined by measuring the length and width of visible subcutaneous tumors. Both of Flt1(1-3)-Fc (A40) and Flt1D2.Flk1D3.FcΔC1(a) significantly reduced the growth of tumors formed by HT-1080 and C6 cells. The results of these experiments are shown in Figure 39 and Figure 40.

Example 31: The Effect of VEGF165 and Modified Flt Receptors in Female Reproductive System

The stereotypic pattern of vascular remodeling which occur in the uterus and ovary over the course of the reproductive cycle has been well characterized, making these tissues particularly well suited to the study of mechanisms which regulate angiogenesis, vascular

remodeling and vascular regression. Indeed, *in situ* hybridization studies in the reproductive tissues provided the first clear evidence that VEGF acts as a mediator of physiological angiogenesis in mature rodents, as well as humans and non-human primates (Phillips et al, 1990; Ravindranath et al, 1992; Shweiki et al, 1993; Kamat et al, 1995). As cyclic angiogenesis and vascular remodeling are prominent features of the normal ovary and uterus, it is not surprising that abnormal blood vessel growth and/or vascular dysfunction have been found to characterize many pathological conditions which affect these organs. Furthermore, these pathogenic vascular abnormalities are thought to be caused or perpetuated by the dysregulated expression of one or more angiogenic or anti-angiogenic factors, most prominently VEGF.

For example, abnormal angiogenesis is characteristic of polycystic ovary disease, endometriosis and endometrial carcinoma, and in each case VEGF is over expressed in the affected tissue (Kamat et al, 1995; Shifren et al, 1996; Guidi et al, 1996; Donnez et al, 1998). Overexpression of VEGF is also thought to play a pathogenic role in the establishment of systemic vascular hyperpermeability in ovarian hyperstimulation syndrome (McClure et al, 1994; Levin et al, 1998) and preeclampsia (Baker et al, 1995; Sharkey et al, 1996). In addition, VEGF has been implicated as the permeability factor responsible for the production of ascites associated with ovarian carcinoma and other tumors (Senger et al, 1983; Boocock et al, 1995). Agents which effectively neutralize the biological actions of VEGF can reasonably be

anticipated to be of therapeutic benefit in the above and related conditions.

Angiogenesis and vascular remodeling are also hallmarks of blastocyst implantation and placental development (Findlay, 1986). VEGF is strongly expressed both in the maternal decidua and in embryonic trophoblasts, where it is thought to first stimulate expansion and hyperpermeability of the uterine vasculature during the peri-implantation period and subsequently mediate formation of both the maternal and embryonic components of the placental vasculature (Shweiki et al, 1993; Cullinan-Bove and Koos, 1993; Chakraborty et al, 1995; Das et al, 1997). VEGF is also required for luteal angiogenesis and associated progesterone secretion necessary to prepare the uterus for implantation (Ferrara et al, 1998). Thus, agents which inhibit the biological actions of VEGF may prove to be useful as contraceptive agents (by preventing implantation), or as an abortifacients in the early stages of gestation. The latter application might find particular use as a non-surgical intervention for the termination of ectopic pregnancies.

While the expression of VEGF receptors is largely confined to the vascular endothelium in normal reproductive tissues, Flt1 is also expressed by trophoblasts in the placenta in both humans and animals (Clark et al, 1996; He et al, 1999) where it has been proposed to play a role in trophoblast invasion. Interestingly, both Flt1 and KDR (Flk1) are expressed by choriocarcinoma cell line BeWo (Charnock-Jones et al, 1994), and VEGF has been shown to promote DNA synthesis and tyrosine phosphorylation of MAP kinase in these cells. Furthermore, primary and

metastatic ovarian carcinomas not only to express high levels of VEGF, but - in addition to the vascular endothelium - the tumor cells themselves express KDR and/ or Flt1 (Boocock et al, 1995). These findings suggest that VEGF may not only be critically involved in the generation and maintenance of tumor vasculature, but that at least in some tumors of reproductive origin VEGF may subserve an autocrine role, directly supporting the survival and proliferation of the tumor cells. Thus agents which block the actions of VEGF may have particularly beneficial applications to the treatment of tumors of reproductive origin.

Methods and Results

(a) Assessment of VEGF-Induced Uterine Hyperpermeability

Pregnant mare's serum gonadotrophin (PMSG) was injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a surge of estradiol after 2 days which in turn causes an induction of VEGF in the uterus. It is reported that this induction results in hyperpermeability of the uterus and an increase in uterine wet weight 6 hrs. later and, therefore, could potentially be blocked by the modified Flt receptors Flt1(1-3)-Fc (A40), Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a). In this in vivo model, the normal weight of the rat uterus is about 50 mg and this can be induced to 300-350 mg by PMSG. Desiccation of the tissue reveals that this is all water weight. Subcutaneous injection of Flt1(1-3)-Fc (A40),

Flt1D2.Flk1D3.FcΔC1(a) and Flt1D2.VEGFR3D3.FcΔC1(a) at 25mg/kg at 1hr. after PMSG injection results in about a 50% inhibition of the increase in uterine wet weight. Increasing the dose of modified Flt receptor does not further reduce the increase in wet weight suggesting that there is a VEGF-independent component to this model. The results of this experiment are shown in Figure 41.

(a) Assessment of corpus luteum angiogenesis using progesterone as a readout

Pregnant mare's serum gonadotrophin (PMSG) is injected subcutaneously (5 IU) to induce ovulation in prepubertal female rats. This results in a fully functioning corpus luteum containing a dense network of blood vessels after 4 days that allows for the secretion of progesterone into the blood stream in order to prepare the uterus for implantation. The induction of angiogenesis in the corpus luteum requires VEGF; therefore, blocking VEGF would result in a lack of new blood vessels and thus a lack of progesterone secreted into the blood stream. In this in vivo model, resting levels of progesterone are about 5ng/ml and this can be induced to a level of 25-40ng/ml after PMSG. Subcutaneous injection of Flt1(1-3)-Fc (A40) or Flt1D2.Flk1D3.FcΔC1(a) at 25mg/kg or 5mg/kg at 1hr. after PMSG injection results in a complete inhibition of the progesterone induction on day 4. The results of this experiment are shown in Figure 42A-42B.

Example 33: Pharmacokinetic Analysis of Flt1(1-3)-Fc (A40) and Pegylated Flt1(1-3)-Fc

Flt1(1-3)-Fc was PEGylated with either 10kD PEG or 20kD PEG and tested in balb/c mice for their pharmacokinetic profile. Both PEGylated forms of Flt1(1-3)-Fc were found to have much better PK profiles than Flt1(1-3)-Fc (A40), with the Tmax occurring at 24 hrs. for the PEGylated molecules as opposed to 6 hrs. for Flt1(1-3)-Fc (A40).

Example 34: VEGF165 ELISA to Test Affinity of Modified Flt1 Receptor Variants

10pM of VEGF165 was incubated overnight at room temperature with modified Flt1 receptor variants ranging from 160pM to 0.1pM. The modified Flt1 receptor variants used in this experiment were Flt1(1-3)-Fc, Flt1(1-3)-Fc (A40), transiently expressed Flt1D2Flk1D3.FcΔC1(a), transiently expressed Flt1D2VEFGFR3D3-FcΔC1(a), Flt1-(1-3_{NAS})-Fc, Flt1(1-$3_{R->C}$)-Fc and Tie2-Fc. Flt1(1-3_{NAS})-Fc is a modified version of Flt1(1-3)-Fc in which the highly basic amino acid sequence KNKRASVRRR is replaced by NASVNGSR, resulting in the incorporation of two new glycosylation sites and a net reduction of five positive charges, both with the purpose of reducing the unfavorable effects of this sequence on PK. Flt1(1-$3_{R->C}$)-Fc is a modification in which a single arginine (R) residue within the same basic amino acid sequence is changed to a cysteine (C) (KNKRASVRRR ->

KNKCASVRRR) to allow for pegylation at that residue, which could then shield the basic region from exerting its unfavorable effects on PK. After incubation the solution was transferred to a plate containing a capture antibody for VEGF165 (R&D). The amount of free VEGF165 was then determined using an antibody to report free VEGF165. This showed that the modified Flt1 receptor variant with the highest affinity for VEGF165 (determined as the lowest amount of free VEGF165) was Flt1D2Flk1D3.FcΔC1(a), followed by Flt1(1-3)-Fc and Flt1(1-3)-Fc (A40) and then by Flt1(1-$3_{R->C}$)-Fc, Flt1(1-3_{NAS})-Fc and Flt1D2VEFGFR3D3-FcΔC1(a). Tie2Fc has no affinity for VEGF165.

WE CLAIM:

1. An isolated nucleic acid molecule encoding a fusion polypeptide capable of binding a VEGF polypeptide comprising:

(a) a nucleotide sequence encoding a VEGF receptor component operatively linked to

(b) a nucleotide sequence encoding a multimerizing component, wherein the VEGF receptor component is the only VEGF receptor component of the fusion polypeptide and wherein the nucleotide sequence of (a) consists essentially of a nucleotide sequence encoding the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and a nucleotide sequence encoding the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor.

2. The isolated nucleic acid of claim 1 wherein the first VEGF receptor is Flt1.

3. The isolated nucleic acid of claim 1 wherein the second VEGF receptor is Flk1.

4. The isolated nucleic acid of claim 1 wherein the second VEGF receptor is Flt4.

5. The isolated nucleic acid molecule of claim 1, wherein the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

6. The isolated nucleic acid molecule of claim 1, wherein the nucleotide sequence encoding Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the nucleotide sequence encoding Ig domain 3 of the extracellular domain of the second VEGF receptor.

7. The isolated nucleic acid molecule of claim 1, wherein the multimerizing component comprises an immunoglobulin domain.

8. The isolated nucleic acid molecule of claim 1, wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

9. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic acid molecule consists essentially of a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in Figure 21A-21C

(b) the nucleotide sequence set forth in Figure 22A-22C;

(c) the nucleotide sequence set forth in Figure 24A-24C; and

(d) a nucleotide sequence which, as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b) or (c) and which encodes a fusion polypeptide molecule having the biological activity of the modified Flt1 receptor fusion polypeptide.

10. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a modified Flt1 receptor fusion polypeptide, wherein the coding region of the nucleic

acid molecule consists essentially of a nucleotide sequence selected from the group consisting of:

(a) the nucleotide sequence set forth in Figure 13A-13D;

(b) the nucleotide sequence set forth in Figure 14A-14C;

(c) the nucleotide sequence set forth in Figure 15A-15C;

(d) the nucleotide sequence set forth in Figure 16A-16D; and

(e) a nucleotide sequence which as a result of the degeneracy of the genetic code, differs from the nucleotide sequence of (a), (b), (c) or (d) and which encodes a fusion polypeptide molecule having the biological activity of the modified Flt1 receptor fusion polypeptide.

11. A fusion polypeptide encoded by the isolated nucleic acid molecule of claim 1, 2, 3, 4, 9 or 10.

12. A composition capable of binding a VEGF molecule to form a nonfunctional complex comprising a multimer of the fusion polypeptide of claim 10.

13. The composition of claim 12, wherein the multimer is a dimer.

14. The composition of claim 13 and a carrier.

15. A vector which comprises the nucleic acid molecule of claim 1, 2, 3, 4, 9 or 10.

16. An expression vector comprising a nucleic acid molecule of claim 1, 2, 3, 4, 9 or 10 wherein the nucleic acid molecule is operatively linked to an expression control sequence.

17. A host-vector system for the production of a fusion polypeptide which comprises the expression vector of claim 16, in a suitable host cell.

18. The host-vector system of claim 17, wherein the suitable host cell is a bacterial cell, yeast cell, insect cell, or mammalian cell.

19. The host-vector system of claim 17, wherein the suitable host cell is E. coli.

20. The host-vector system of claim 17, wherein the suitable host cell is a COS cell or a CHO cell.

21. A method of producing a fusion polypeptide which comprises growing cells of the host-vector system of claim 17, under conditions permitting production of the fusion polypeptide and recovering the fusion polypeptide so produced.

22. A fusion polypeptide encoded by the nucleic acid sequence set forth in Figure 10A-10D, Figure 21A-21C, Figure 22A-22C, or Figure 24A-24C, which has been modified by acetylation or pegylation.

23. The fusion polypeptide of claim 22 wherein the modification is acetylation.

24. The fusion polypeptide of claim 22 wherein the modification is pegylation.

25. The fusion polypeptide of claim 23 wherein the acetylation is accomplished with at least about a 100 fold molar excess of acetylation reagent.

26. The fusion polypeptide of claim 23 wherein acetylation is accomplished with a molar excess of acetylation reagent ranging from at least about a 10 fold molar excess to about a 100 fold molar excess.

27. The fusion polypeptide of claim 24 wherein the pegylation is 10K or 20K PEG.

28. A method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal fusion polypeptide of claim ~~10~~ 11.

29. The method of claim 28, wherein the mammal is a human.

30. The method of claim 29, wherein the fusion polypeptide is acetylated.

31. The method of claim 29, wherein the fusion polypeptide is pegylated.

32. The fusion polypeptide of claims ~~10~~ 11 which specifically binds the VEGF receptor ligand VEGF.

33. A method of blocking blood vessel growth in a human comprising administering an effective amount of the fusion polypeptide of claim ~~10~~ 11.

34. A method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide of claim ~~10~~ 11.

35. The method of claim 34, wherein the mammal is a human.

36. The method of claim 34, used to attenuate or prevent tumor growth in a human.

37. The method of claim 34, used to attenuate or prevent edema in a human.

38. The method of claim 34, used to attenuate or prevent ascites formation in a human.

39. The method of claim 37, wherein the edema is brain edema.

40. The method of claim 38, wherein the ascites is ovarian cancer associated ascites.

41. A fusion polypeptide capable of binding a VEGF polypeptide comprising:

(a a VEGF receptor component operatively linked to

(b) a multimerizing component,

wherein the VEGF receptor component is the only VEGF receptor component in the fusion polypeptide and consists essentially of the amino acid sequence of Ig domain 2 of the extracellular domain of a first VEGF receptor and the amino acid sequence of Ig domain 3 of the extracellular domain of a second VEGF receptor.

42. The fusion polypeptide of claim 41 wherein the first VEGF receptor is Flt1.

43. The fusion polypeptide of claim 41 wherein the second VEGF receptor is Flk1.

44. The fusion polypeptide of claim 41 wherein the second VEGF receptor is Flt4.

45. The fusion polypeptide claim 41, wherein amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is upstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

46. The fusion polypeptide of claim 41, wherein the amino acid sequence of Ig domain 2 of the extracellular domain of the first VEGF receptor is downstream of the amino acid sequence of Ig domain 3 of the extracellular domain of the second VEGF receptor.

47. The fusion polypeptide of claim 41, wherein the multimerizing component comprises an immunoglobulin domain.

48. The fusion polypeptide of claim 41, wherein the immunoglobulin domain is selected from the group consisting of the Fc domain of IgG, the heavy chain of IgG, and the light chain of IgG.

49. A fusion polypeptide consisting essentially of an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence is selected from the group consisting of:

(a) the amino acid sequence set forth in Figure 21A-21C

(b) the amino acid sequence set forth in Figure 22A-22C; and

(c) the amino acid sequence set forth in Figure 24A-24C.

50. A fusion polypeptide comprising an amino acid sequence of a modified Flt1 receptor, wherein the amino acid sequence is selected from the group consisting of:

(a) the amino acid sequence set forth in Figure 13A-13D;

(b) the amino acid sequence set forth in Figure 14A-14C;

(c) the amino acid sequence set forth in Figure 15A-15C; and

(d) the amino acid sequence set forth in Figure 16A-16D;

51. A method of decreasing or inhibiting plasma leakage in a mammal comprising administering to the mammal fusion polypeptide of claim 41, 42, 43, 44, 49 or 50.

52. A method of inhibiting VEGF receptor ligand activity in a mammal comprising administering to the mammal an effective amount of the fusion polypeptide of claim 41, 42, 43, 44, 49 or 50.

Fig.1.

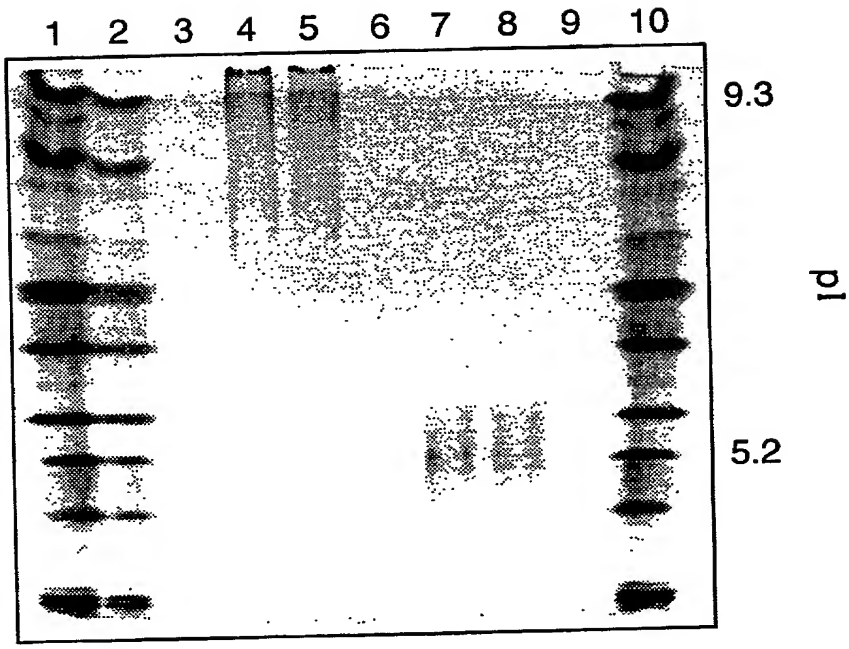

1 2 3 4 5 6 7 8 9 10
9.3
5.2
pI

Fig.2.

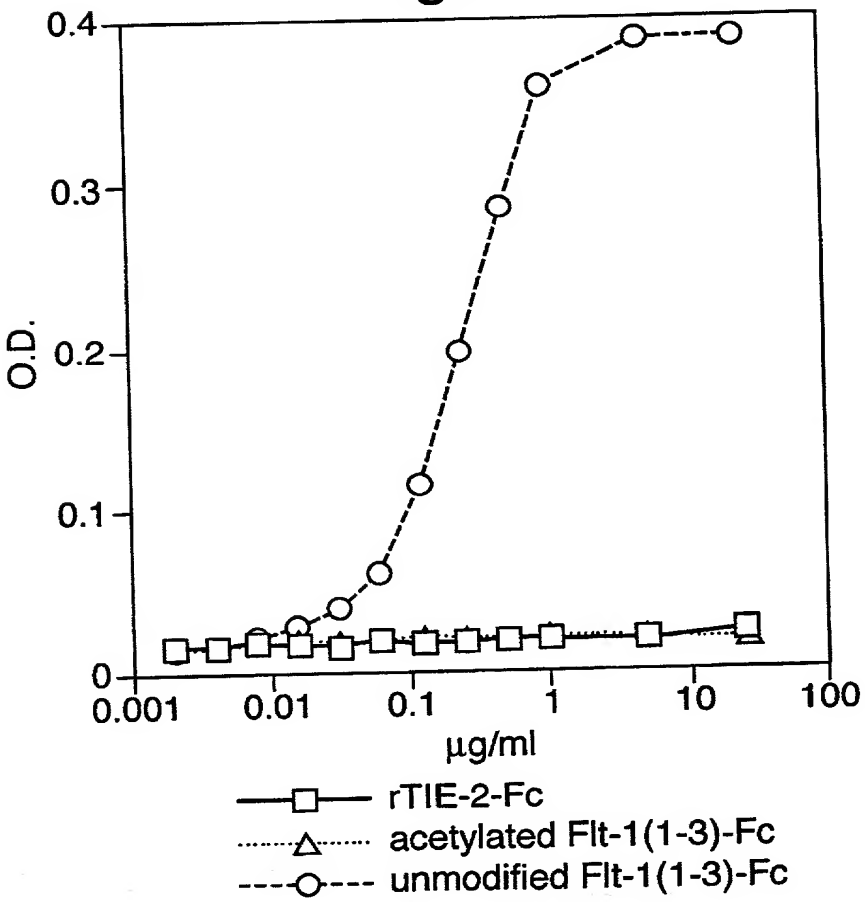
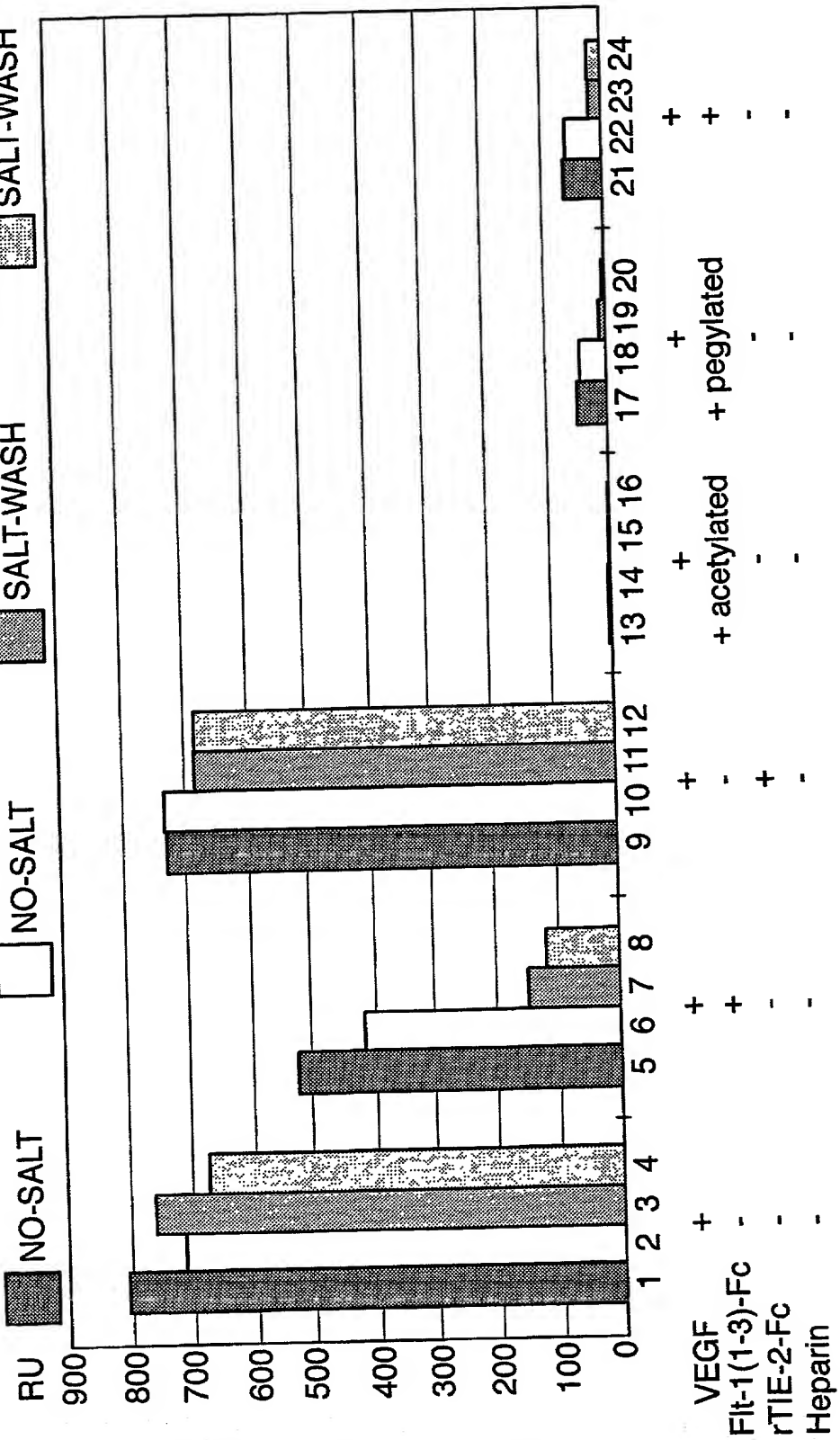

0.4
0.3
0.2
0.1
0
O.D.
0.001
0.01
0.1
1
10
100
µg/ml
rTIE-2-Fc
acetylated Flt-1(1-3)-Fc
unmodified Flt-1(1-3)-Fc

Fig.3.


NO-SALT
NO-SALT
SALT-WASH
SALT-WASH
RU
900
800
700
600
500
400
300
200
100
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
VEGF + + + + - - - - + + + + - - - -
Flt-1(1-3)-Fc - - - - + + - - - - + + - - - -
rTIE-2-Fc - - - - - - + + - - - - + + - -
Heparin - - - - - - - - + + + + + + + +
+ acetylated
+ pegylated

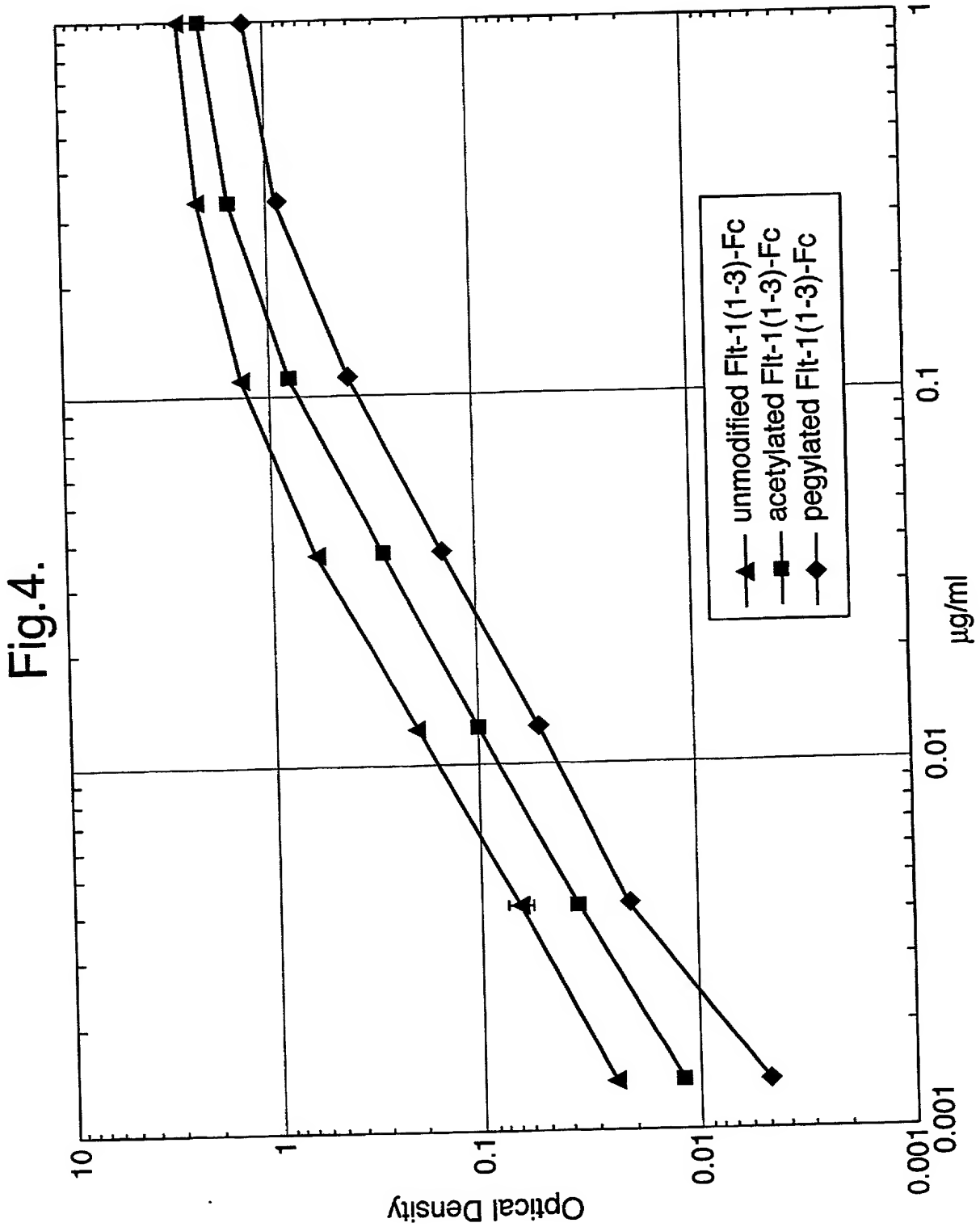

Optical Density
10
1
0.1
0.01
0.001
0.001
0.01
0.1
1
µg/ml
unmodified Flt-1(1-3)-Fc
acetylated Flt-1(1-3)-Fc
pegylated Flt-1(1-3)-Fc

Fig.4.

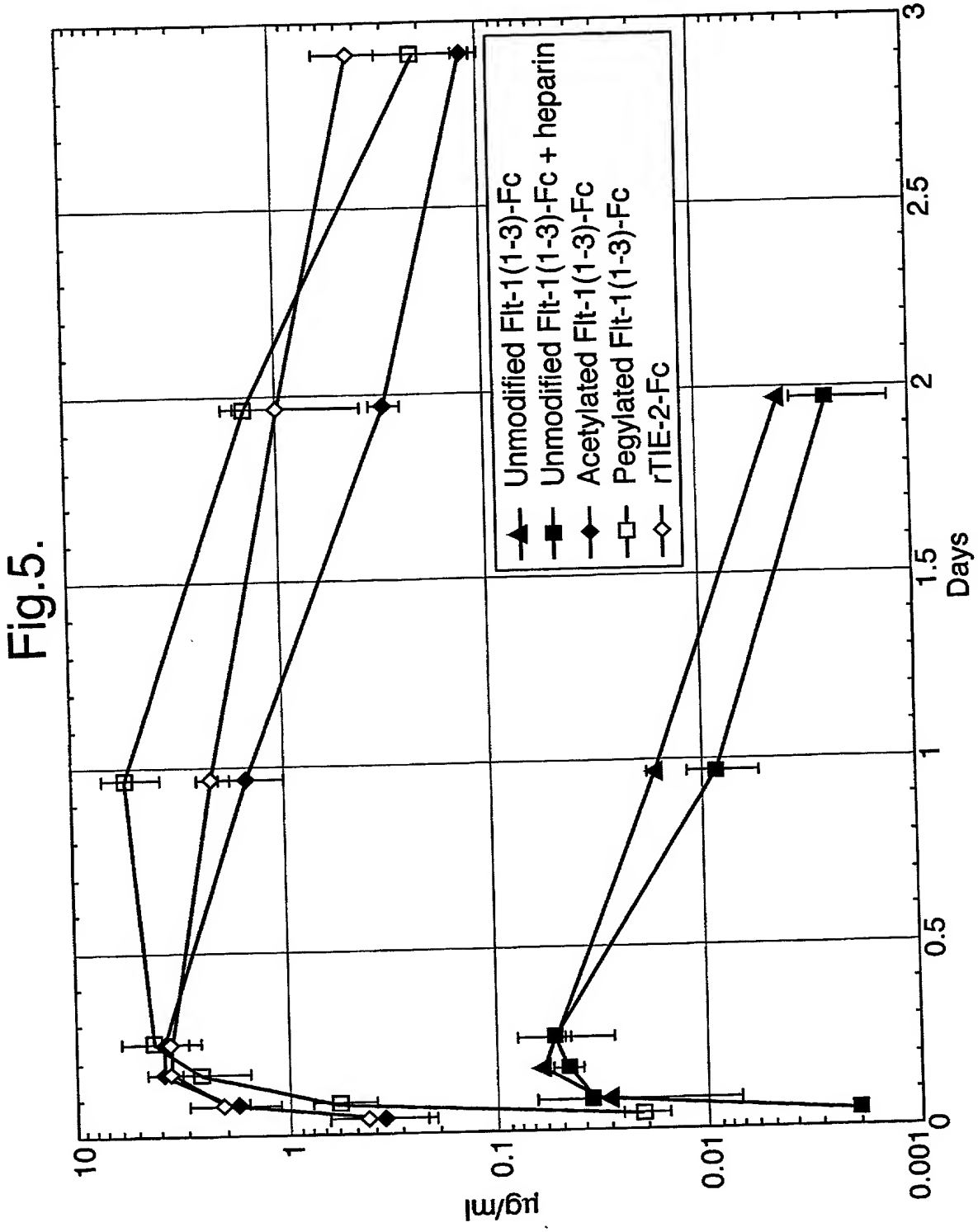

Unmodified Flt-1(1-3)-Fc
Unmodified Flt-1(1-3)-Fc + heparin
Acetylated Flt-1(1-3)-Fc
Pegylated Flt-1(1-3)-Fc
rTIE-2-Fc
μg/ml
10
1
0.1
0.01
0.001
Days
0
0.5
1
1.5
2
2.5
3

Fig.5.

Fig.6A.

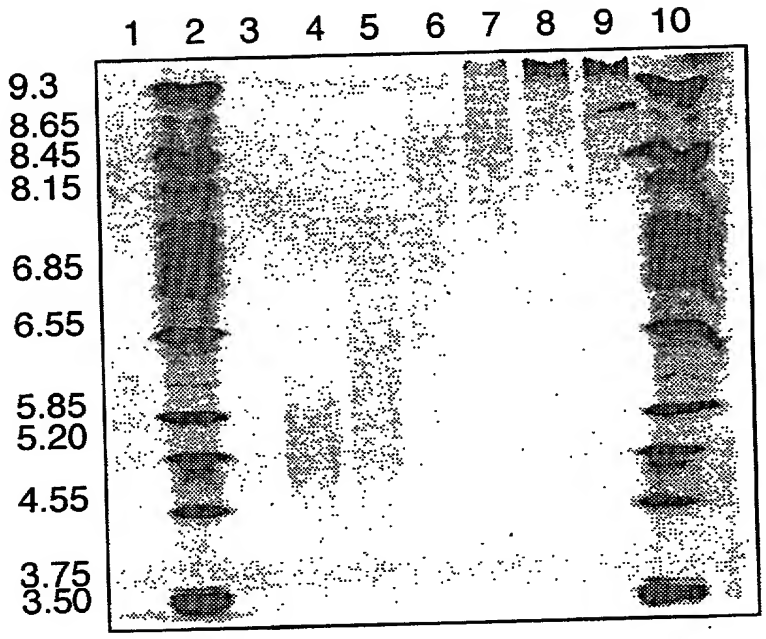

1 2 3 4 5 6 7 8 9 10
9.3
8.65
8.45
8.15
6.85
6.55
5.85
5.20
4.55
3.75
3.50

Fig.6B.

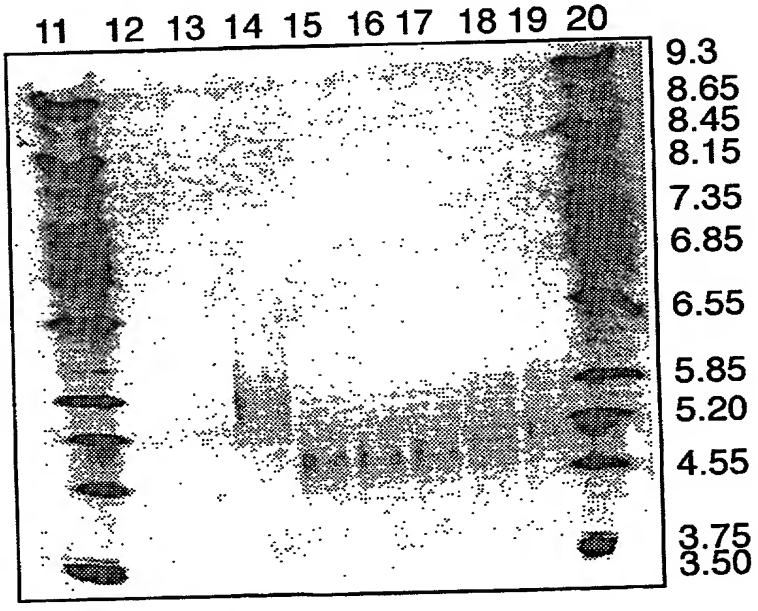

11 12 13 14 15 16 17 18 19 20
9.3
8.65
8.45
8.15
7.35
6.85
6.55
5.85
5.20
4.55
3.75
3.50

Fig.7.

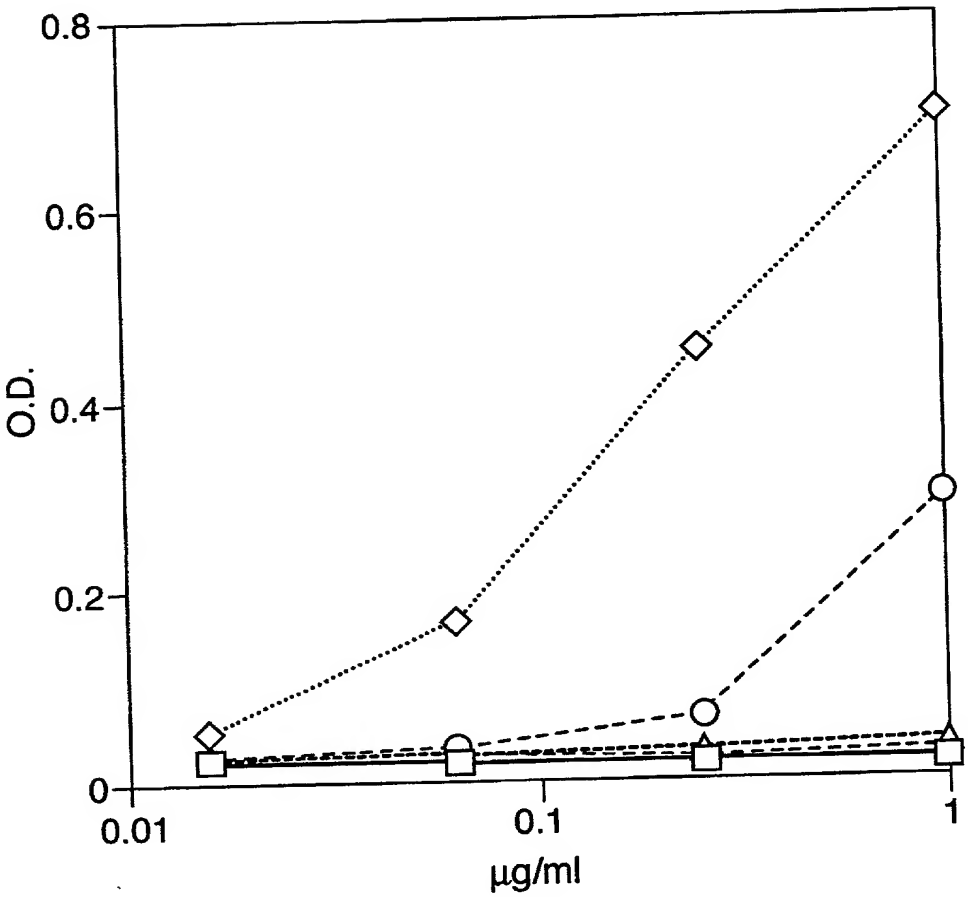
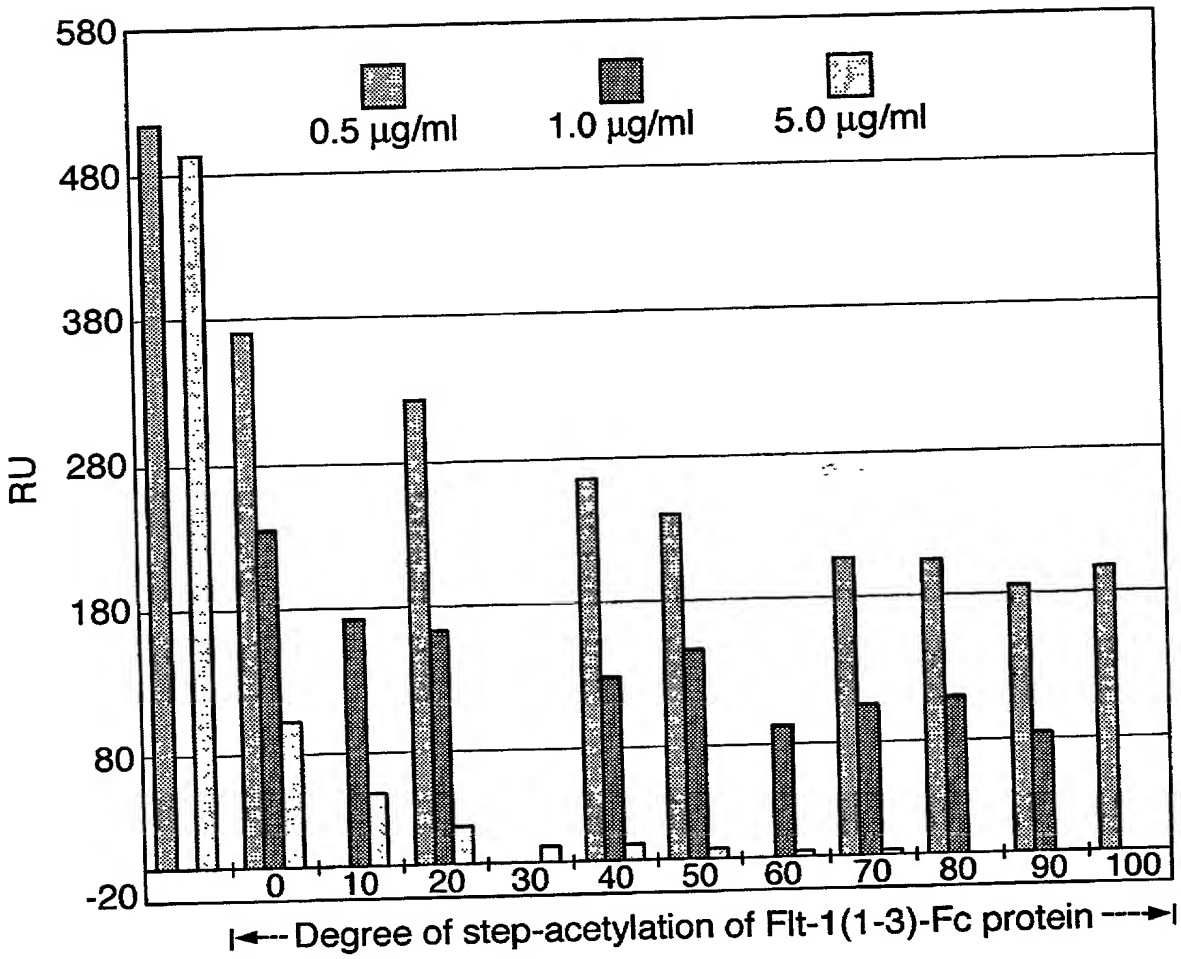

0.8
0.6
0.4
0.2
0
O.D.
0.01
0.1
1
µg/ml
rTIE-2-Fc
unmodified Flt-1(1-3)-Fc
acetylated Flt-1(1-3)-Fc (10X)
acetylated Flt-1(1-3)-Fc (20X)
acetylated Flt-1(1-3)-Fc (30X)

Fig.8.



VEGF 0.2 µg/ml	+	+	+	+	+	+	+	+	+	+	+	+
Unmodified Flt-1(1-3) -Fc	-	+	-	-	-	-	-	-	-	-	-	-
Acetylated Flt-1(1-3) -Fc	-	-	+	+	+	+	+	+	+	+	+	+

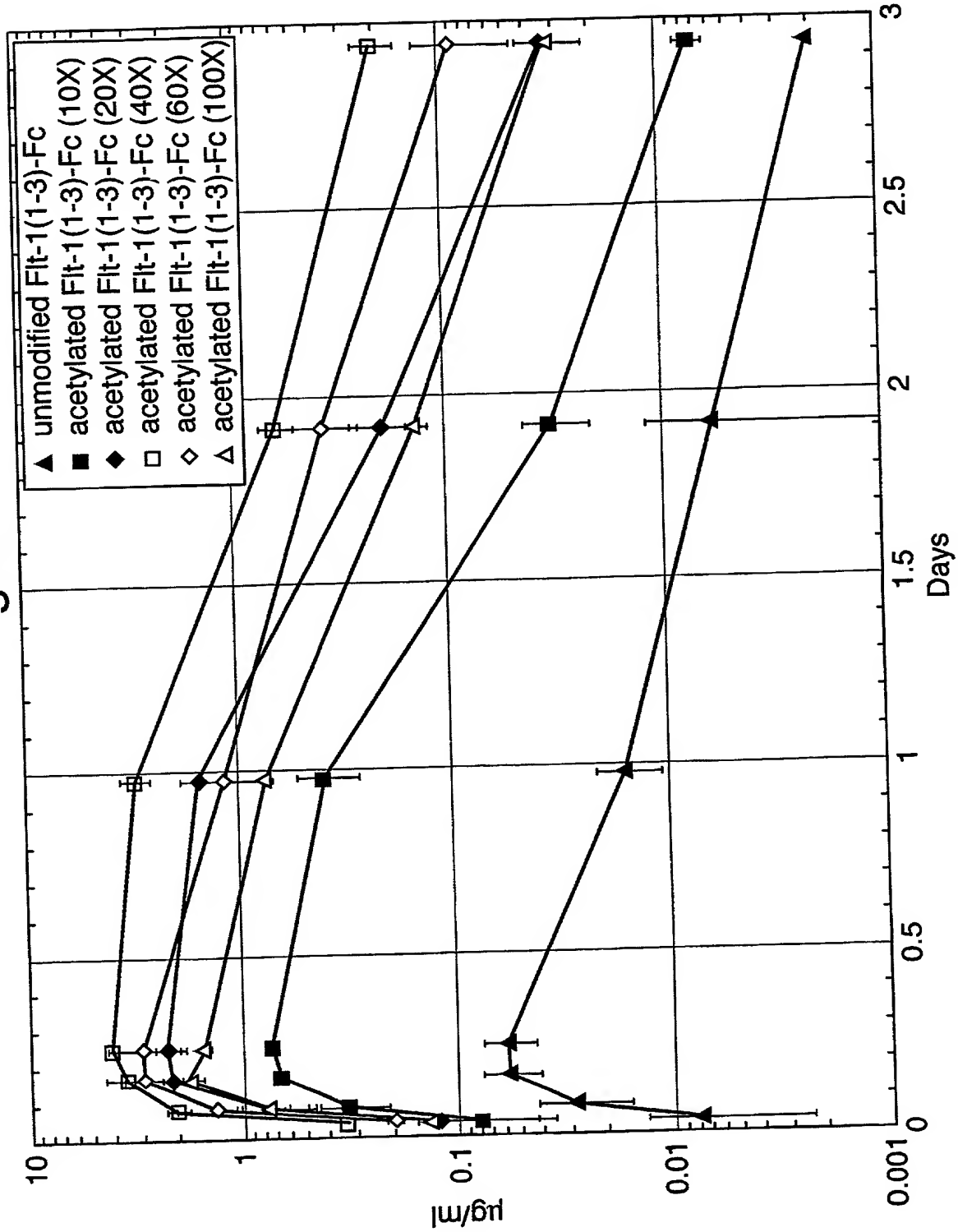

unmodified Flt-1(1-3)-Fc
acetylated Flt-1(1-3)-Fc (10X)
acetylated Flt-1(1-3)-Fc (20X)
acetylated Flt-1(1-3)-Fc (40X)
acetylated Flt-1(1-3)-Fc (60X)
acetylated Flt-1(1-3)-Fc (100X)
10
1
0.1
0.01
0.001
µg/ml
0
0.5
1
1.5
2
2.5
3
Days

Fig.9.

Fig.10A.

```
          10         20         30         40         50         60
     *    *    *    *    *    *    *    *    *    *    *    *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>

          70         80         90        100        110        120
     *    *    *    *    *    *    *    *    *    *    *    *
ACA GGA TCT AGT TCA GGT TCA AAA TTA AAA GAT CCT GAA CTG AGT TTA AAA GGC ACC CAG
TGT CCT AGA TCA AGT CCA AGT TTT AAT TTT CTA GGA CTT GAC TCA AAT TTT CCG TGG GTC
Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro Glu Leu Ser Leu Lys Gly Thr Gln>

         130        140        150        160        170        180
     *    *    *    *    *    *    *    *    *    *    *    *
CAC ATC ATG CAA GCA GGC CAG ACA CTG CAT CTC CAA TGC AGG GGG GAA GCA GCC CAT AAA
GTG TAG TAC GTT CGT CCG GTC TGT GAC GTA GAG GTT ACG TCC CCC CTT CGT CGG GTA TTT
His Ile Met Gln Ala Gly Gln Thr Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys>

         190        200        210        220        230        240
     *    *    *    *    *    *    *    *    *    *    *    *
TGG TCT TTG CCT GAA ATG GTG AGT AAG GAA AGC GAA AGG CTG AGC ATA ACT AAA TCT GCC
ACC AGA AAC GGA CTT TAC CAC TCA TTC CTT TCG CTT TCC GAC TCG TAT TGA TTT AGA CGG
Trp Ser Leu Pro Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala>

         250        260        270        280        290        300
     *    *    *    *    *    *    *    *    *    *    *    *
TGT GGA AGA AAT GGC AAA CAA TTC TGC AGT ACT TTA ACC TTG AAC ACA GCT CAA GCA AAC
ACA CCT TCT TTA CCG TTT GTT AAG ACG TCA TGA AAT TGG AAC TTG TGT CGA GTT CGT TTG
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr Ala Gln Ala Asn>

         310        320        330        340        350        360
     *    *    *    *    *    *    *    *    *    *    *    *
CAC ACT GGC TTC TAC AGC TGC AAA TAT CTA GCT GTA CCT ACT TCA AAG AAG AAG GAA ACA
GTG TGA CCG AAG ATG TCG ACG TTT ATA GAT CGA CAT GGA TGA AGT TTC TTC TTC CTT TGT
His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val Pro Thr Ser Lys Lys Lys Glu Thr>

         370        380        390        400        410        420
     *    *    *    *    *    *    *    *    *    *    *    *
GAA TCT GCA ATC TAT ATA TTT ATT AGT GAT ACA GGT AGA CCT TTC GTA GAG ATG TAC AGT
CTT AGA CGT TAG ATA TAT AAA TAA TCA CTA TGT CCA TCT GGA AAG CAT CTC TAC ATG TCA
Glu Ser Ala Ile Tyr Ile Phe Ile Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser>

         430        440        450        460        470        480
     *    *    *    *    *    *    *    *    *    *    *    *
GAA ATC CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT
CTT TAG GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val>

         490        500        510        520        530        540
     *    *    *    *    *    *    *    *    *    *    *    *
ACG TCA CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT
TGC AGT GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp>
```

Fig.10B.

```
          550         560         570         580         590         600
     *    *    *    *    *    *    *    *    *    *    *    *
GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA
CCT TTT GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT
Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys>

          610         620         630         640         650         660
     *    *    *    *    *    *    *    *    *    *    *    *
GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT
CTT TAT CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA
Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr>

          670         680         690         700         710         720
     *    *    *    *    *    *    *    *    *    *    *    *
CTC ACA CAT CGA CAA ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC
GAG TGT GTA GCT GTT TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG
Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val>

          730         740         750         760         770         780
     *    *    *    *    *    *    *    *    *    *    *    *
AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG
TTT AAT GAA TCT CCG GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr>

          790         800         810         820         830         840
     *    *    *    *    *    *    *    *    *    *    *    *
AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA AAA AAT AAG AGA GCT TCC GTA AGG CGA
TCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TTT TTA TTC TCT CGA AGG CAT TCC GCT
Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg>

          850         860         870         880         890         900
     *    *    *    *    *    *    *    *    *    *    *    *
CGA ATT GAC CAA AGC AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA
GCT TAA CTG GTT TCG TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT
Arg Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys>

          910         920         930         940         950         960
     *    *    *    *    *    *    *    *    *    *    *    *
ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA
TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT
Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys>

          970         980         990        1000        1010        1020
     *    *    *    *    *    *    *    *    *    *    *    *
TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT
AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys>

         1030        1040        1050        1060        1070        1080
     *    *    *    *    *    *    *    *    *    *    *    *
GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC
CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG
Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val>
```

Fig.10C.

```
        1090        1100        1110        1120        1130        1140
     *     *     *     *     *     *     *     *     *     *     *     *
TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA
AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT
Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr>

        1150        1160        1170        1180        1190        1200
     *     *     *     *     *     *     *     *     *     *     *     *
TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC
ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp>

        1210        1220        1230        1240        1250        1260
     *     *     *     *     *     *     *     *     *     *     *     *
GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC
CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr>

        1270        1280        1290        1300        1310        1320
     *     *     *     *     *     *     *     *     *     *     *     *
CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG
GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC
Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys>

        1330        1340        1350        1360        1370        1380
     *     *     *     *     *     *     *     *     *     *     *     *
TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA
ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT
Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys>

        1390        1400        1410        1420        1430        1440
     *     *     *     *     *     *     *     *     *     *     *     *
GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG
CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys>

        1450        1460        1470        1480        1490        1500
     *     *     *     *     *     *     *     *     *     *     *     *
AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG
TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu>

        1510        1520        1530        1540        1550        1560
     *     *     *     *     *     *     *     *     *     *     *     *
TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC
ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG
Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser>

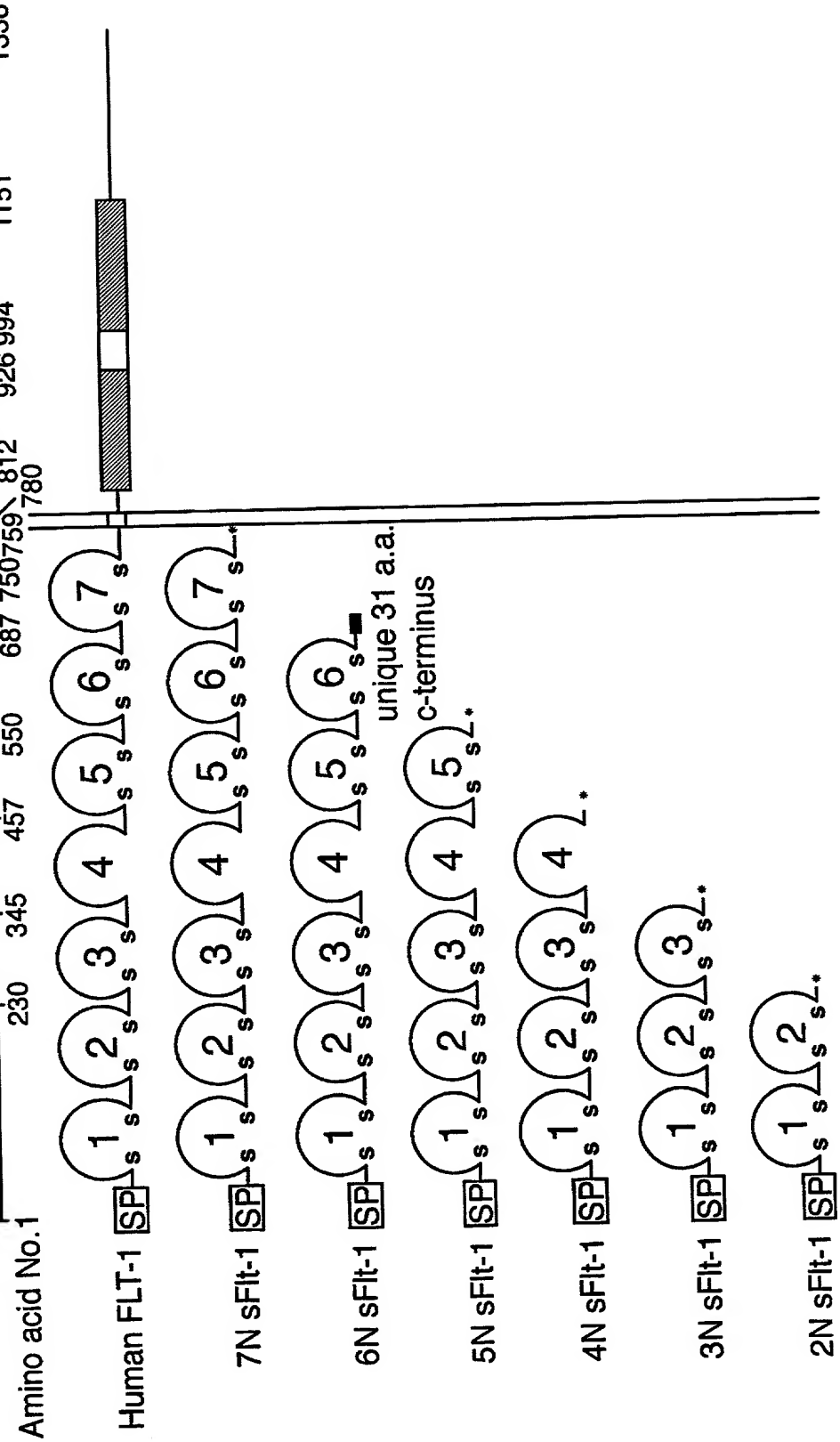
        1570        1580        1590        1600        1610        1620
     *     *     *     *     *     *     *     *     *     *     *     *
GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG
CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC
Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly>
```

Fig.10D.

```
        1630        1640        1650        1660        1670        1680
     *     *     *     *     *     *     *     *     *     *     *     *
AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC
TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG
Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser>

        1690        1700
     *     *     *     *
CTC TCC CTG TCT CCG GGT AAA TGA
GAG AGG GAC AGA GGC CCA TTT ACT
Leu Ser Leu Ser Pro Gly Lys ***>
```

Fig.11.


Extracellular ligand binding domains
Transmembrane
Intracellular kinase domain
2N
3N
4N
5N
6N
7N
230
345
457
550
687
750
759
780
812
926
994
1151
1338
Amino acid No.1
Human FLT-1
7N sFlt-1
6N sFlt-1
5N sFlt-1
4N sFlt-1
3N sFlt-1
2N sFlt-1
SP
unique 31 a.a.
c-terminus

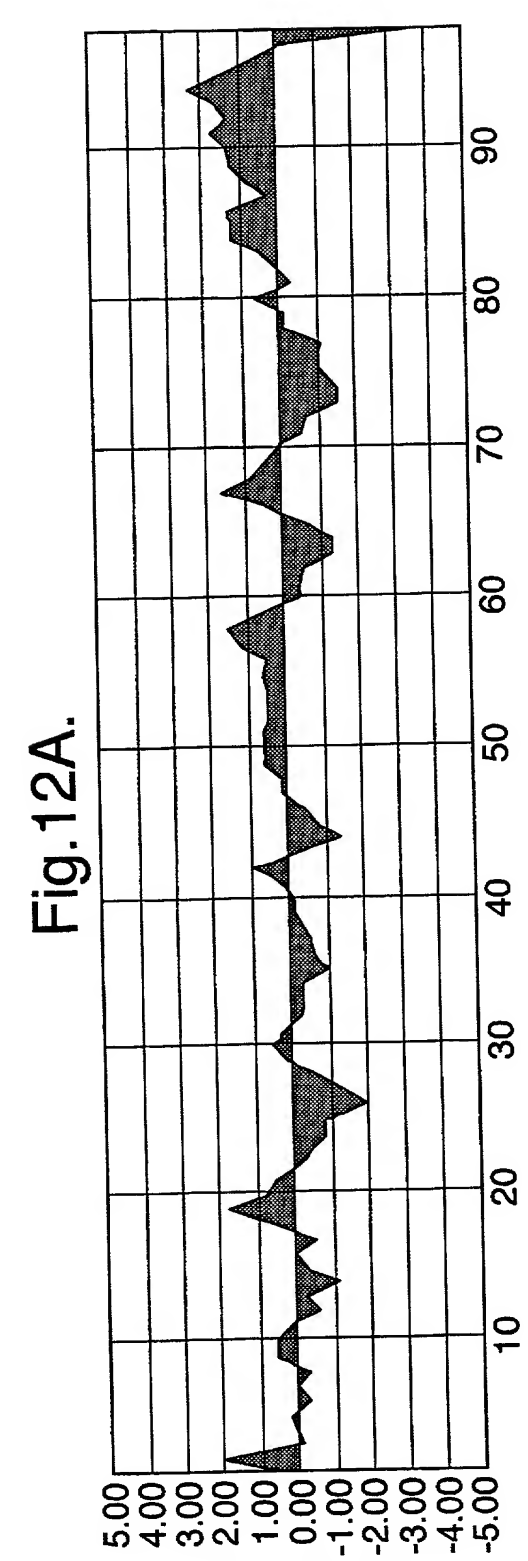


Fig.12A.

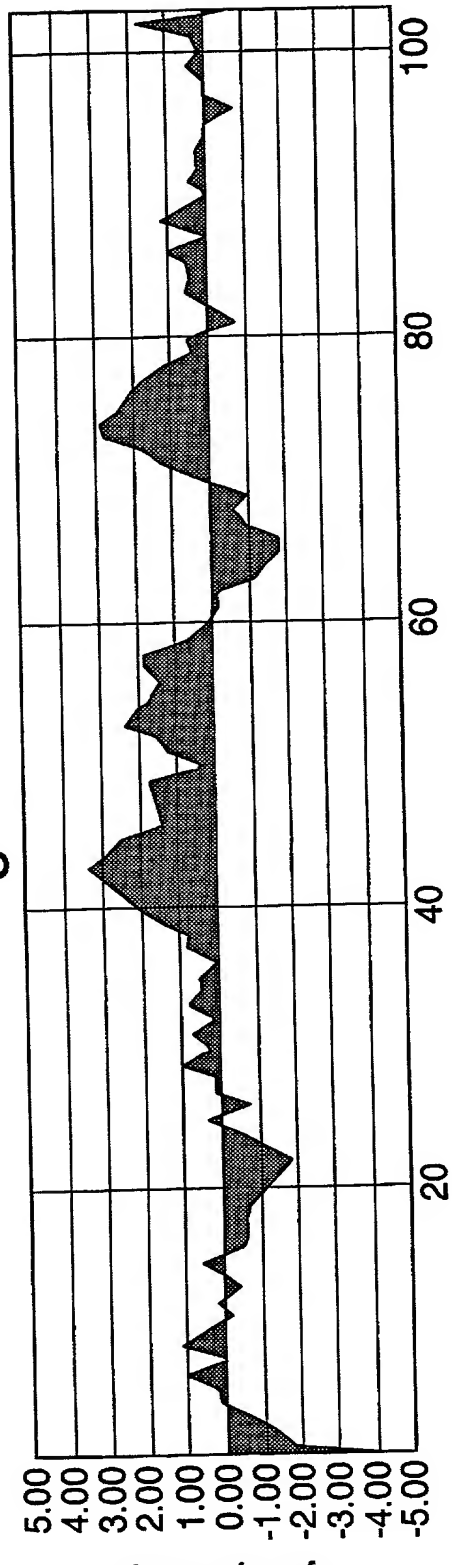


Fig.12B.

Fig.13A.

```
          10          20          30          40          50          60
     *    *    *    *    *    *    *    *    *    *    *    *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>

          70          80          90          100         110         120
     *    *    *    *    *    *    *    *    *    *    *    *
ACA GGA TCT AGT TCA GGT TCA AAA TTA AAA GAT CCT GAA CTG AGT TTA AAA GGC ACC CAG
TGT CCT AGA TCA AGT CCA AGT TTT AAT TTT CTA GGA CTT GAC TCA AAT TTT CCG TGG GTC
Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro Glu Leu Ser Leu Lys Gly Thr Gln>

          130         140         150         160         170         180
     *    *    *    *    *    *    *    *    *    *    *    *
CAC ATC ATG CAA GCA GGC CAG ACA CTG CAT CTC CAA TGC AGG GGG GAA GCA GCC CAT AAA
GTG TAG TAC GTT CGT CCG GTC TGT GAC GTA GAG GTT ACG TCC CCC CTT CGT CGG GTA TTT
His Ile Met Gln Ala Gly Gln Thr Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys>

          190         200         210         220         230         240
     *    *    *    *    *    *    *    *    *    *    *    *
TGG TCT TTG CCT GAA ATG GTG AGT AAG GAA AGC GAA AGG CTG AGC ATA ACT AAA TCT GCC
ACC AGA AAC GGA CTT TAC CAC TCA TTC CTT TCG CTT TCC GAC TCG TAT TGA TTT AGA CGG
Trp Ser Leu Pro Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala>

          250         260         270         280         290         300
     *    *    *    *    *    *    *    *    *    *    *    *
TGT GGA AGA AAT GGC AAA CAA TTC TGC AGT ACT TTA ACC TTG AAC ACA GCT CAA GCA AAC
ACA CCT TCT TTA CCG TTT GTT AAG ACG TCA TGA AAT TGG AAC TTG TGT CGA GTT CGT TTG
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr Ala Gln Ala Asn>

          310         320         330         340         350         360
     *    *    *    *    *    *    *    *    *    *    *    *
CAC ACT GGC TTC TAC AGC TGC AAA TAT CTA GCT GTA CCT ACT TCA AAG AAG AAG GAA ACA
GTG TGA CCG AAG ATG TCG ACG TTT ATA GAT CGA CAT GGA TGA AGT TTC TTC TTC CTT TGT
His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val Pro Thr Ser Lys Lys Lys Glu Thr>

          370         380         390         400         410         420
     *    *    *    *    *    *    *    *    *    *    *    *
GAA TCT GCA ATC TAT ATA TTT ATT AGT GAT ACA GGT AGA CCT TTC GTA GAG ATG TAC AGT
CTT AGA CGT TAG ATA TAT AAA TAA TCA CTA TGT CCA TCT GGA AAG CAT CTC TAC ATG TCA
Glu Ser Ala Ile Tyr Ile Phe Ile Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser>

          430         440         450         460         470         480
     *    *    *    *    *    *    *    *    *    *    *    *
GAA ATC CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT
CTT TAG GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val>

          490         500         510         520         530         540
     *    *    *    *    *    *    *    *    *    *    *    *
ACG TCA CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT
TGC AGT GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp>
```

Fig.13B.

```
          550         560         570         580         590         600
     *    *    *    *    *    *    *    *    *    *    *    *
GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA
CCT TTT GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT
Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys>

          610         620         630         640         650         660
     *    *    *    *    *    *    *    *    *    *    *    *
GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT
CTT TAT CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA
Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr>

          670         680         690         700         710         720
     *    *    *    *    *    *    *    *    *    *    *    *
CTC ACA CAT CGA CAA ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC
GAG TGT GTA GCT GTT TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG
Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val>

          730         740         750         760         770         780
     *    *    *    *    *    *    *    *    *    *    *    *
AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG
TTT AAT GAA TCT CCG GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr>

          790         800         810         820         830         840
     *    *    *    *    *    *    *    *    *    *    *    *
AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA ATT GAC CAA AGC AAT TCC CAT GCC AAC
TCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TAA CTG GTT TCG TTA AGG GTA CGG TTG
Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn>

          850         860         870         880         890         900
     *    *    *    *    *    *    *    *    *    *    *    *
ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT
TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA
Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr>

          910         920         930         940         950         960
     *    *    *    *    *    *    *    *    *    *    *    *
TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT
ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA
Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp>

          970         980         990         1000        1010        1020
     *    *    *    *    *    *    *    *    *    *    *    *
AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA
TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT
Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro>

          1030        1040        1050        1060        1070        1080
     *    *    *    *    *    *    *    *    *    *    *    *
GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC
CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG
Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr>
```

Fig.13C.

```
        1090        1100        1110        1120        1130        1140
     *     *     *     *     *     *     *     *     *     *     *     *
CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC
GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG
Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp>

        1150        1160        1170        1180        1190        1200
     *     *     *     *     *     *     *     *     *     *     *     *
CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG
GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC
Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys>

        1210        1220        1230        1240        1250        1260
     *     *     *     *     *     *     *     *     *     *     *     *
CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC
GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG
Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His>

        1270        1280        1290        1300        1310        1320
     *     *     *     *     *     *     *     *     *     *     *     *
CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC
GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG
Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala>

        1330        1340        1350        1360        1370        1380
     *     *     *     *     *     *     *     *     *     *     *     *
CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC
GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG
Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr>

        1390        1400        1410        1420        1430        1440
     *     *     *     *     *     *     *     *     *     *     *     *
CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA
GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT
Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys>

        1450        1460        1470        1480        1490        1500
     *     *     *     *     *     *     *     *     *     *     *     *
GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC
CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG
Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn>

        1510        1520        1530        1540        1550        1560
     *     *     *     *     *     *     *     *     *     *     *     *
TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC
ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG
Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu>

        1570        1580        1590        1600        1610        1620
     *     *     *     *     *     *     *     *     *     *     *     *
ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG
TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC
Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu>
```

Fig.13D.

```
        1630        1640        1650        1660        1670
     *     *     *     *     *     *     *     *     *     *
GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT
Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
```

Fig.14A.

```
        10        20        30        40        50        60
         *    *    *    *    *    *    *    *    *    *    *    *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>

        70        80        90       100       110       120
         *    *    *    *    *    *    *    *    *    *    *    *
ACA GGA TCT AGT TCC GGA GGT AGA CCT TTC GTA GAG ATG TAC AGT GAA ATC CCC GAA ATT
TGT CCT AGA TCA AGG CCT CCA TCT GGA AAG CAT CTC TAC ATG TCA CTT TAG GGG CTT TAA
Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile>

       130       140       150       160       170       180
         *    *    *    *    *    *    *    *    *    *    *    *
ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT ACG TCA CCT AAC ATC
TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA TGC AGT GGA TTG TAG
Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile>

       190       200       210       220       230       240
         *    *    *    *    *    *    *    *    *    *    *    *
ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT GGA AAA CGC ATA ATC
TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA CCT TTT GCG TAT TAG
Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile>

       250       260       270       280       290       300
         *    *    *    *    *    *    *    *    *    *    *    *
TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA GAA ATA GGG CTT CTG
ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT CTT TAT CCC GAA GAC
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu>

       310       320       330       340       350       360
         *    *    *    *    *    *    *    *    *    *    *    *
ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTC ACA CAT CGA CAA
TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA GAG TGT GTA GCT GTT
Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln>

       370       380       390       400       410       420
         *    *    *    *    *    *    *    *    *    *    *    *
ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC AAA TTA CTT AGA GGC
TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG TTT AAT GAA TCT CCG
Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly>

       430       440       450       460       470       480
         *    *    *    *    *    *    *    *    *    *    *    *
CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG AGA GTT CAA ATG ACC
GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC TCT CAA GTT TAC TGG
His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr>

       490       500       510       520       530       540
         *    *    *    *    *    *    *    *    *    *    *    *
TGG AGT TAC CCT GAT GAA ATT GAC CAA AGC AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT
ACC TCA ATG GGA CTA CTT TAA CTG GTT TCG TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA
Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val>
```

Fig.14B.

```
        550         560         570         580         590         600
         *     *     *     *     *     *     *     *     *     *     *     *
CTT ACT ATT GAC AAA ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT
GAA TGA TAA CTG TTT TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA
Leu Thr Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser>

        610         620         630         640         650         660
         *     *     *     *     *     *     *     *     *     *     *     *
GGA CCA TCA TTC AAA TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC
CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG
Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly>

        670         680         690         700         710         720
         *     *     *     *     *     *     *     *     *     *     *     *
GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG
CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC
Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu>

        730         740         750         760         770         780
         *     *     *     *     *     *     *     *     *     *     *     *
GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG
CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg>

        790         800         810         820         830         840
         *     *     *     *     *     *     *     *     *     *     *     *
ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC
TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe>

        850         860         870         880         890         900
         *     *     *     *     *     *     *     *     *     *     *     *
AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG
TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC
Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln>

        910         920         930         940         950         960
         *     *     *     *     *     *     *     *     *     *     *     *
TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT
ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA
Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn>

        970         980         990        1000        1010        1020
         *     *     *     *     *     *     *     *     *     *     *     *
GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC
CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr>

       1030        1040        1050        1060        1070        1080
         *     *     *     *     *     *     *     *     *     *     *     *
ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG
TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg>
```

Fig.14C.

```
          1090        1100        1110        1120        1130        1140
     *    *    *    *    *    *    *    *    *    *    *    *
GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC
CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG
Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser>

          1150        1160        1170        1180        1190        1200
     *    *    *    *    *    *    *    *    *    *    *    *
GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT
CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA
Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro>

          1210        1220        1230        1240        1250        1260
     *    *    *    *    *    *    *    *    *    *    *    *
CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC
GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser>

          1270        1280        1290        1300        1310        1320
     *    *    *    *    *    *    *    *    *    *    *    *
AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC
TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG
Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His>

          1330        1340        1350
     *    *    *    *    *    *    *
TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT
Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys ***>
```

Fig.15A.

```
          10          20          30          40          50          60
     *    *    *    *    *    *    *    *    *    *    *    *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>

          70          80          90          100         110         120
     *    *    *    *    *    *    *    *    *    *    *    *
ACA GGA TCT AGT TCC GGA GGT AGA CCT TTC GTA GAG ATG TAC AGT GAA ATC CCC GAA ATT
TGT CCT AGA TCA AGG CCT CCA TCT GGA AAG CAT CTC TAC ATG TCA CTT TAG GGG CTT TAA
Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile>

          130         140         150         160         170         180
     *    *    *    *    *    *    *    *    *    *    *    *
ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT ACG TCA CCT AAC ATC
TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA TGC AGT GGA TTG TAG
Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile>

          190         200         210         220         230         240
     *    *    *    *    *    *    *    *    *    *    *    *
ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT GGA AAA CGC ATA ATC
TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA CCT TTT GCG TAT TAG
Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile>

          250         260         270         280         290         300
     *    *    *    *    *    *    *    *    *    *    *    *
TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA GAA ATA GGG CTT CTG
ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT CTT TAT CCC GAA GAC
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu>

          310         320         330         340         350         360
     *    *    *    *    *    *    *    *    *    *    *    *
ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTC ACA CAT CGA CAA
TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA GAG TGT GTA GCT GTT
Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln>

          370         380         390         400         410         420
     *    *    *    *    *    *    *    *    *    *    *    *
ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC AAA TTA CTT AGA GGC
TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG TTT AAT GAA TCT CCG
Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly>

          430         440         450         460         470         480
     *    *    *    *    *    *    *    *    *    *    *    *
CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG AGA GTT CAA ATG ACC
GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC TCT CAA GTT TAC TGG
His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr>

          490         500         510         520         530         540
     *    *    *    *    *    *    *    *    *    *    *    *
TGG AGT TAC CCT GAT GAA AAA AAT AAG AGA GCT TCC GTA AGG CGA CGA ATT GAC CAA AGC
ACC TCA ATG GGA CTA CTT TTT TTA TTC TCT CGA AGG CAT TCC GCT GCT TAA CTG GTT TCG
Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg Arg Ile Asp Gln Ser>
```

Fig.15B.

```
          550         560         570         580         590         600
     *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA ATG CAG AAC AAA GAC
TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT TAC GTC TTG TTT CTG
Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys Asp>

          610         620         630         640         650         660
     *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA TCT GTT AAC ACC TCA
TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT AGA CAA TTG TGG AGT
Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser>

          670         680         690         700         710         720
     *    *    *    *    *    *    *    *    *    *    *    *    *    *
GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT GAC AAA ACT CAC ACA
CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA CTG TTT TGA GTG TGT
Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr>

          730         740         750         760         770         780
     *    *    *    *    *    *    *    *    *    *    *    *    *    *
TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA
ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro>

          790         800         810         820         830         840
     *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC
TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp>

          850         860         870         880         890         900
     *    *    *    *    *    *    *    *    *    *    *    *    *    *
GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT
CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA
Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His>

          910         920         930         940         950         960
     *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC
TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG
Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val>

          970         980         990         1000        1010        1020
     *    *    *    *    *    *    *    *    *    *    *    *    *    *
CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC
GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn>

          1030        1040        1050        1060        1070        1080
     *    *    *    *    *    *    *    *    *    *    *    *    *    *
AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA
TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu>
```

Fig.15C.

```
          1090      1100      1110      1120      1130      1140
        *     *     *     *     *     *     *     *     *     *
CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG
GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC
Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu>

          1150      1160      1170      1180      1190      1200
        *     *     *     *     *     *     *     *     *     *
ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG
TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC
Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly>

          1210      1220      1230      1240      1250      1260
        *     *     *     *     *     *     *     *     *     *
CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC
GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe>

          1270      1280      1290      1300      1310      1320
        *     *     *     *     *     *     *     *     *     *
CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC
GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys>

          1330      1340      1350      1360      1370      1380
        *     *     *     *     *     *     *     *     *     *
TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG
AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC
Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro>

        *
GGT AAA TGA
CCA TTT ACT
Gly Lys ***>
```

Fig.16A.

```
          10          20          30          40          50          60
     *    *    *    *    *    *    *    *    *    *    *    *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser Cys Leu Leu Leu>

          70          80          90          100         110         120
     *    *    *    *    *    *    *    *    *    *    *    *
ACA GGA TCT AGT TCA GGT TCA AAA TTA AAA GAT CCT GAA CTG AGT TTA AAA GGC ACC CAG
TGT CCT AGA TCA AGT CCA AGT TTT AAT TTT CTA GGA CTT GAC TCA AAT TTT CCG TGG GTC
Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro Glu Leu Ser Leu Lys Gly Thr Gln>

          130         140         150         160         170         180
     *    *    *    *    *    *    *    *    *    *    *    *
CAC ATC ATG CAA GCA GGC CAG ACA CTG CAT CTC CAA TGC AGG GGG GAA GCA GCC CAT AAA
GTG TAG TAC GTT CGT CCG GTC TGT GAC GTA GAG GTT ACG TCC CCC CTT CGT CGG GTA TTT
His Ile Met Gln Ala Gly Gln Thr Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys>

          190         200         210         220         230         240
     *    *    *    *    *    *    *    *    *    *    *    *
TGG TCT TTG CCT GAA ATG GTG AGT AAG GAA AGC GAA AGG CTG AGC ATA ACT AAA TCT GCC
ACC AGA AAC GGA CTT TAC CAC TCA TTC CTT TCG CTT TCC GAC TCG TAT TGA TTT AGA CGG
Trp Ser Leu Pro Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala>

          250         260         270         280         290         300
     *    *    *    *    *    *    *    *    *    *    *    *
TGT GGA AGA AAT GGC AAA CAA TTC TGC AGT ACT TTA ACC TTG AAC ACA GCT CAA GCA AAC
ACA CCT TCT TTA CCG TTT GTT AAG ACG TCA TGA AAT TGG AAC TTG TGT CGA GTT CGT TTG
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr Ala Gln Ala Asn>

          310         320         330         340         350         360
     *    *    *    *    *    *    *    *    *    *    *    *
CAC ACT GGC TTC TAC AGC TGC AAA TAT CTA GCT GTA CCT ACT TCA AAG AAG AAG GAA ACA
GTG TGA CCG AAG ATG TCG ACG TTT ATA GAT CGA CAT GGA TGA AGT TTC TTC TTC CTT TGT
His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val Pro Thr Ser Lys Lys Lys Glu Thr>

          370         380         390         400         410         420
     *    *    *    *    *    *    *    *    *    *    *    *
GAA TCT GCA ATC TAT ATA TTT ATT AGT GAT ACA GGT AGA CCT TTC GTA GAG ATG TAC AGT
CTT AGA CGT TAG ATA TAT AAA TAA TCA CTA TGT CCA TCT GGA AAG CAT CTC TAC ATG TCA
Glu Ser Ala Ile Tyr Ile Phe Ile Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser>

          430         440         450         460         470         480
     *    *    *    *    *    *    *    *    *    *    *    *
GAA ATC CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT
CTT TAG GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val>

          490         500         510         520         530         540
     *    *    *    *    *    *    *    *    *    *    *    *
ACG TCA CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT
TGC AGT GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp>
```

Fig.16B.

```
          550         560         570         580         590         600
     *    *    *    *    *    *    *    *    *    *    *    *
GGA AAA CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA
CCT TTT GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT
Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys>

          610         620         630         640         650         660
     *    *    *    *    *    *    *    *    *    *    *    *
GAA ATA GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT
CTT TAT CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA
Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr>

          670         680         690         700         710         720
     *    *    *    *    *    *    *    *    *    *    *    *
CTC ACA CAT CGA CAA ACC AAT ACA ATC ATA GAT GTC CAA ATA AGC ACA CCA CGC CCA GTC
GAG TGT GTA GCT GTT TGG TTA TGT TAG TAT CTA CAG GTT TAT TCG TGT GGT GCG GGT CAG
Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val>

          730         740         750         760         770         780
     *    *    *    *    *    *    *    *    *    *    *    *
AAA TTA CTT AGA GGC CAT ACT CTT GTC CTC AAT TGT ACT GCT ACC ACT CCC TTG AAC ACG
TTT AAT GAA TCT CCG GTA TGA GAA CAG GAG TTA ACA TGA CGA TGG TGA GGG AAC TTG TGC
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr>

          790         800         810         820         830         840
     *    *    *    *    *    *    *    *    *    *    *    *
AGA GTT CAA ATG ACC TGG AGT TAC CCT GAT GAA AAA AAT AAG AAC GCT TCC GTA AGG CGA
TCT CAA GTT TAC TGG ACC TCA ATG GGA CTA CTT TTT TTA TTC TTG CGA AGG CAT TCC GCT
Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys Asn Lys Asn Ala Ser Val Arg Arg>

          850         860         870         880         890         900
     *    *    *    *    *    *    *    *    *    *    *    *
CGA ATT GAC CAA AGC AAT TCC CAT GCC AAC ATA TTC TAC AGT GTT CTT ACT ATT GAC AAA
GCT TAA CTG GTT TCG TTA AGG GTA CGG TTG TAT AAG ATG TCA CAA GAA TGA TAA CTG TTT
Arg Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys>

          910         920         930         940         950         960
     *    *    *    *    *    *    *    *    *    *    *    *
ATG CAG AAC AAA GAC AAA GGA CTT TAT ACT TGT CGT GTA AGG AGT GGA CCA TCA TTC AAA
TAC GTC TTG TTT CTG TTT CCT GAA ATA TGA ACA GCA CAT TCC TCA CCT GGT AGT AAG TTT
Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys>

          970         980         990         1000        1010        1020
     *    *    *    *    *    *    *    *    *    *    *    *
TCT GTT AAC ACC TCA GTG CAT ATA TAT GAT AAA GCA GGC CCG GGC GAG CCC AAA TCT TGT
AGA CAA TTG TGG AGT CAC GTA TAT ATA CTA TTT CGT CCG GGC CCG CTC GGG TTT AGA ACA
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys>

          1030        1040        1050        1060        1070        1080
     *    *    *    *    *    *    *    *    *    *    *    *
GAC AAA ACT CAC ACA TGC CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC
CTG TTT TGA GTG TGT ACG GGT GGC ACG GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG
Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val>
```

Fig.16C.

```
        1090        1100        1110        1120        1130        1140
    *   *   *   *   *   *   *   *   *   *   *   *
TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA
AAG GAG AAG GGG GGT TTT GGG TTC CTG TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT
Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr>

        1150        1160        1170        1180        1190        1200
    *   *   *   *   *   *   *   *   *   *   *   *
TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC
ACG CAC CAC CAC CTG CAC TCG GTG CTT CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp>

        1210        1220        1230        1240        1250        1260
    *   *   *   *   *   *   *   *   *   *   *   *
GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC
CCG CAC CTC CAC GTA TTA CGG TTC TGT TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr>

        1270        1280        1290        1300        1310        1320
    *   *   *   *   *   *   *   *   *   *   *   *
CGT GTG GTC AGC GTC CTC ACC GTC CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG
GCA CAC CAG TCG CAG GAG TGG CAG GAC GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC
Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys>

        1330        1340        1350        1360        1370        1380
    *   *   *   *   *   *   *   *   *   *   *   *
TGC AAG GTC TCC AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA
ACG TTC CAG AGG TTG TTT CGG GAG GGT CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT
Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys>

        1390        1400        1410        1420        1430        1440
    *   *   *   *   *   *   *   *   *   *   *   *
GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG
CCC GTC GGG GCT CTT GGT GTC CAC ATG TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys>

        1450        1460        1470        1480        1490        1500
    *   *   *   *   *   *   *   *   *   *   *   *
AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG
TTG GTC CAG TCG GAC TGG ACG GAC CAG TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu>

        1510        1520        1530        1540        1550        1560
    *   *   *   *   *   *   *   *   *   *   *   *
TGG GAG AGC AAT GGG CAG CCG GAG AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC
ACC CTC TCG TTA CCC GTC GGC CTC TTG TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG
Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser>

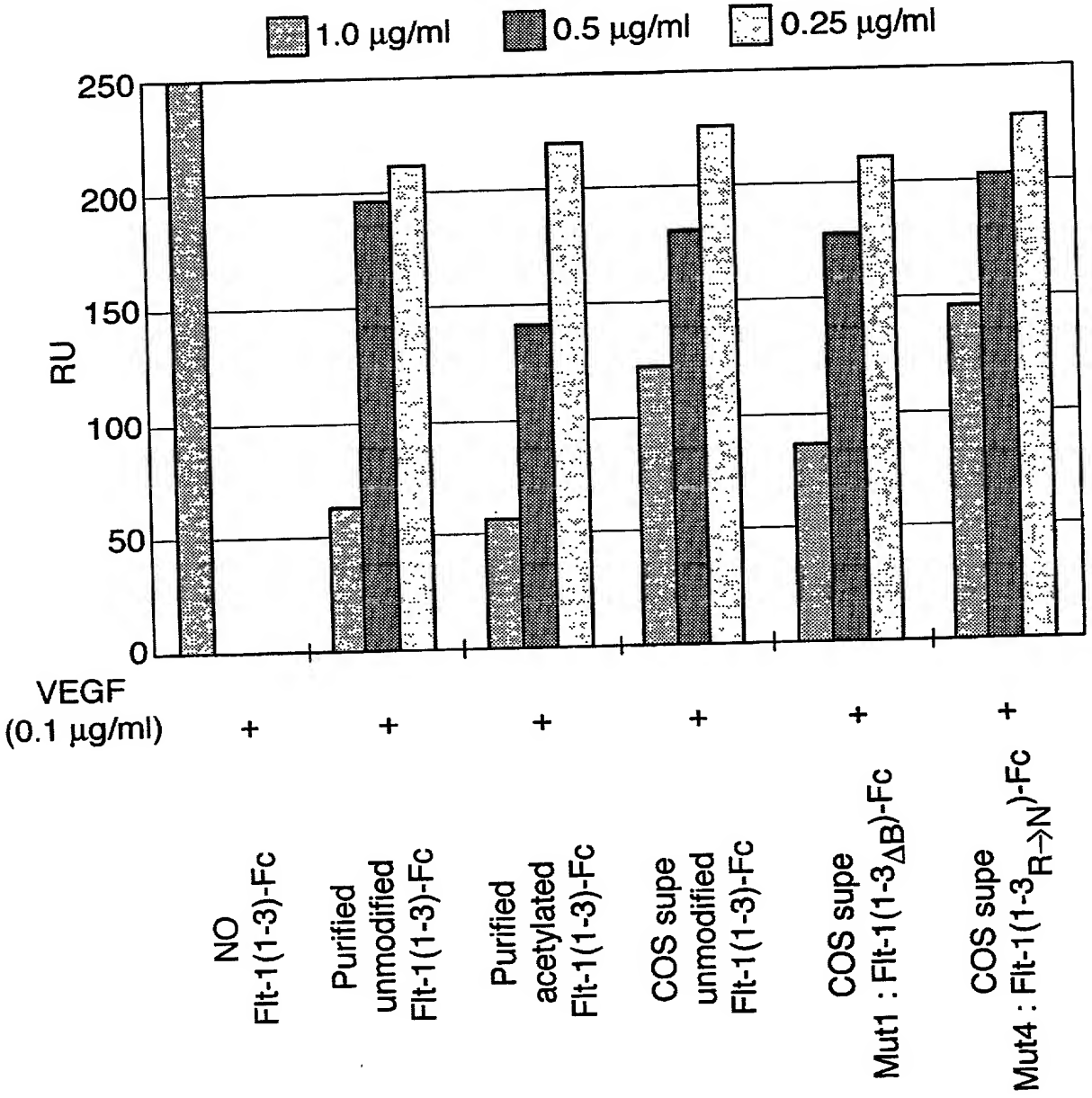
        1570        1580        1590        1600        1610        1620
    *   *   *   *   *   *   *   *   *   *   *   *
GAC GGC TCC TTC TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG
CTG CCG AGG AAG AAG GAG ATG TCG TTC GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC
Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly>
```

Fig.16D.

```
          1630          1640          1650          1660          1670          1680
      *     *     *     *     *     *     *     *     *     *     *     *
AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC
TTG CAG AAG AGT ACG AGG CAC TAC GTA CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG
Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser>

          1690          1700
      *     *     *     *
CTC TCC CTG TCT CCG GGT AAA TGA
GAG AGG GAC AGA GGC CCA TTT ACT
Leu Ser Leu Ser Pro Gly Lys ***>
```

Fig.17.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150
100
50
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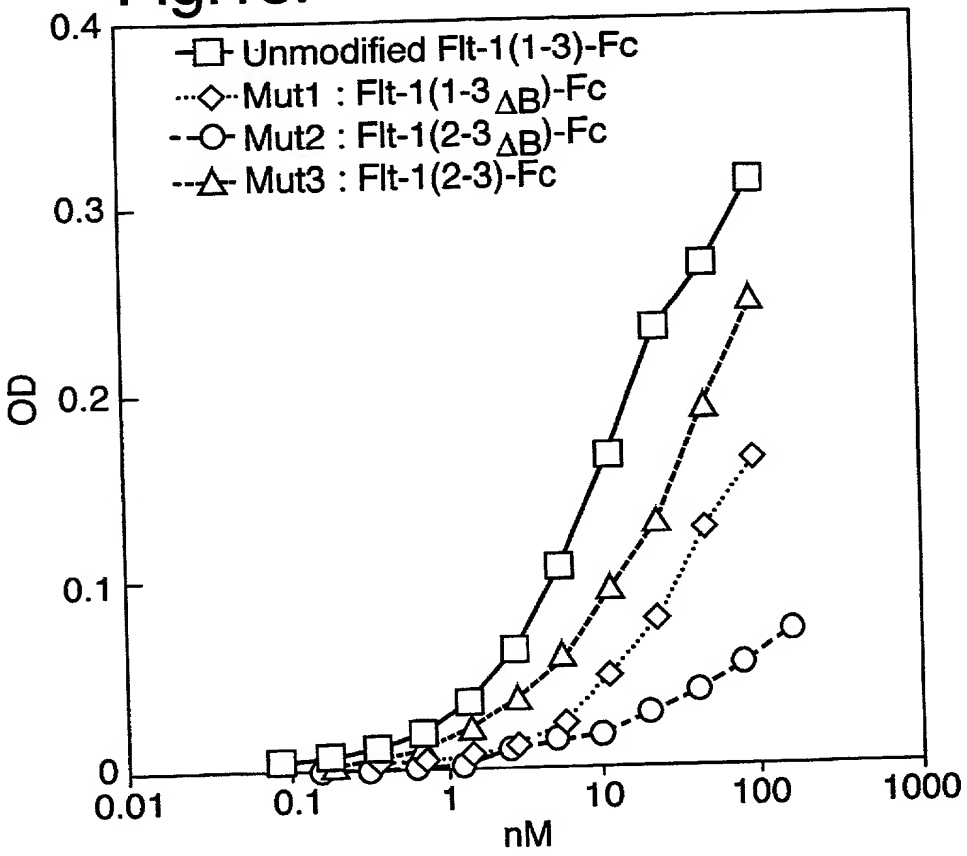
VEGF
(0.1 µg/ml)
+
+
+
+
+
+
NO Flt-1(1-3)-Fc
Purified unmodified Flt-1(1-3)-Fc
Purified acetylated Flt-1(1-3)-Fc
COS supe unmodified Flt-1(1-3)-Fc
COS supe Mut1 : Flt-1(1-3ΔB)-Fc
COS supe Mut4 : Flt-1(1-3R→N)-Fc

Fig.18.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Mut1 : Flt-1(1-3 ΔB)-Fc
Mut2 : Flt-1(2-3 ΔB)-Fc
Mut3 : Flt-1(2-3)-Fc
OD
0.4
0.3
0.2
0.1
0
0.01
0.1
1
10
100
1000
nM

Fig.19.

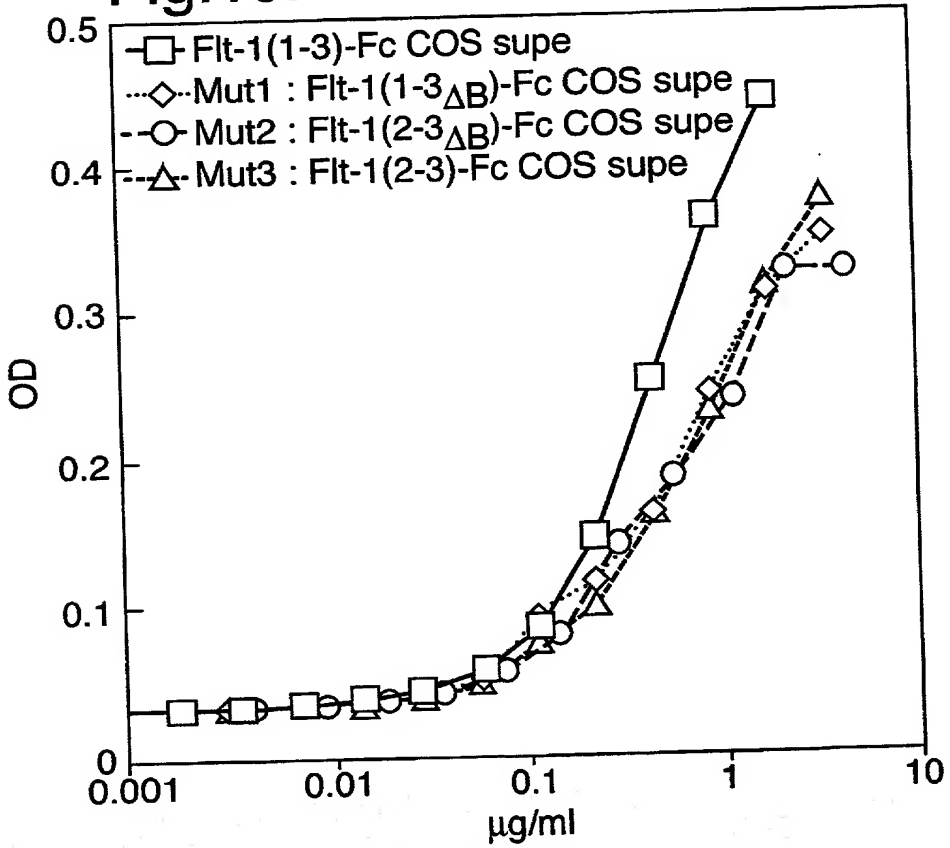

Flt-1(1-3)-Fc COS supe
Mut1 : Flt-1(1-3 ΔB)-Fc COS supe
Mut2 : Flt-1(2-3 ΔB)-Fc COS supe
Mut3 : Flt-1(2-3)-Fc COS supe
OD
0.5
0.4
0.3
0.2
0.1
0
0.001
0.01
0.1
1
10
μg/ml

Fig.20.

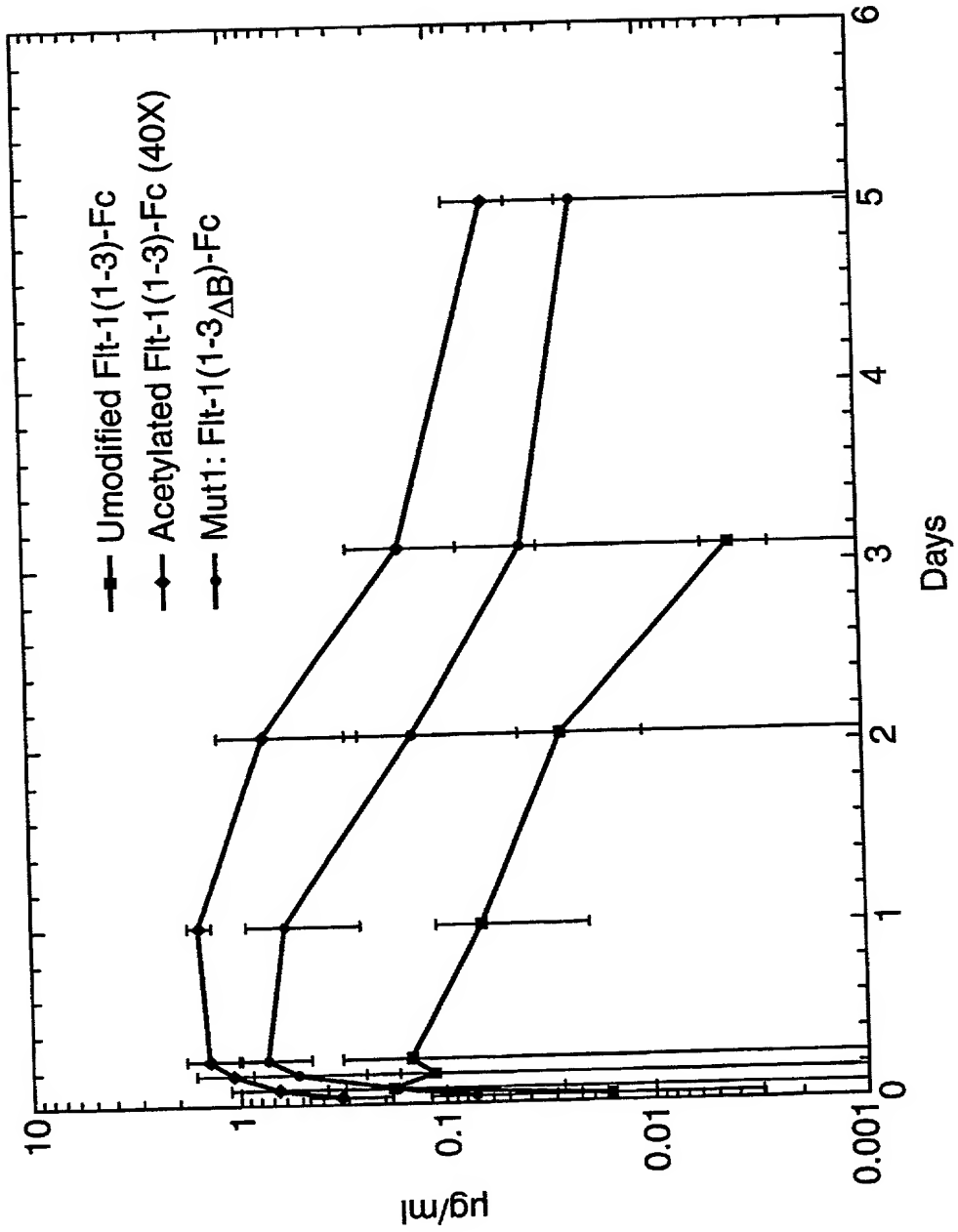

Umodified Flt-1(1-3)-Fc
Acetylated Flt-1(1-3)-Fc (40X)
Mut1: Flt-1(1-3ΔB)-Fc
10
1
0.1
0.01
0.001
µg/ml
0
1
2
3
4
5
6
Days

Fig.21A.

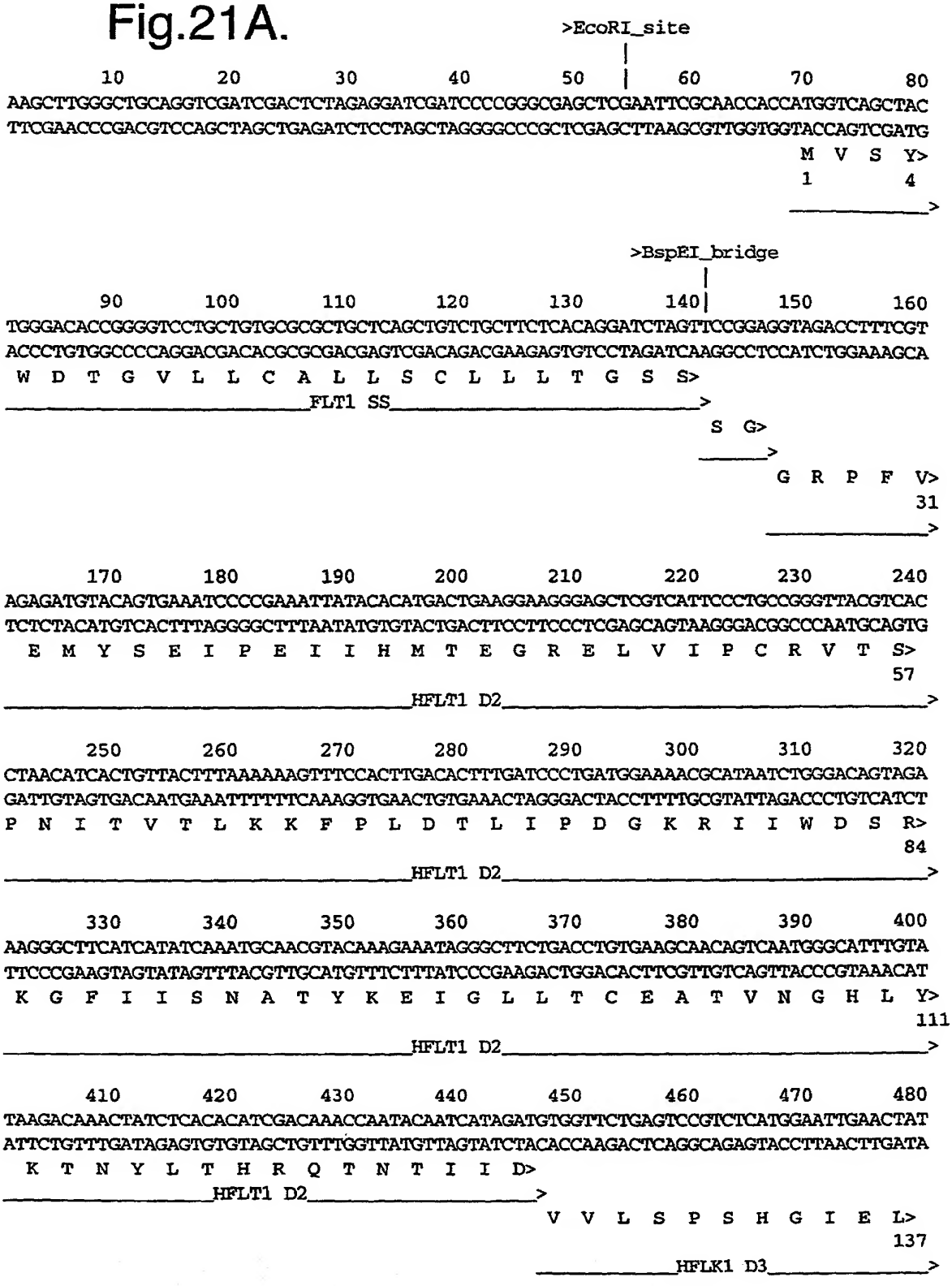
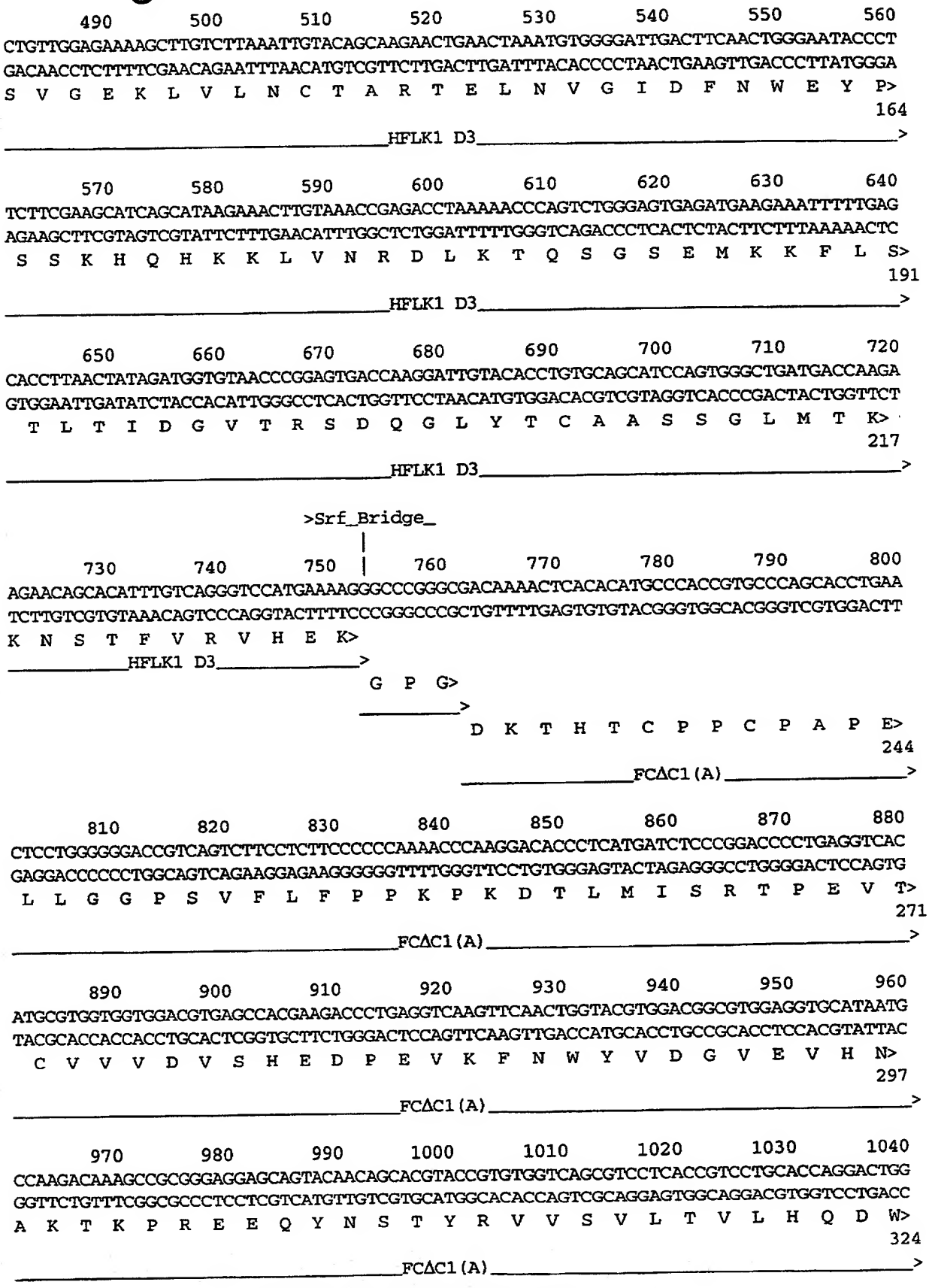


Fig.21B.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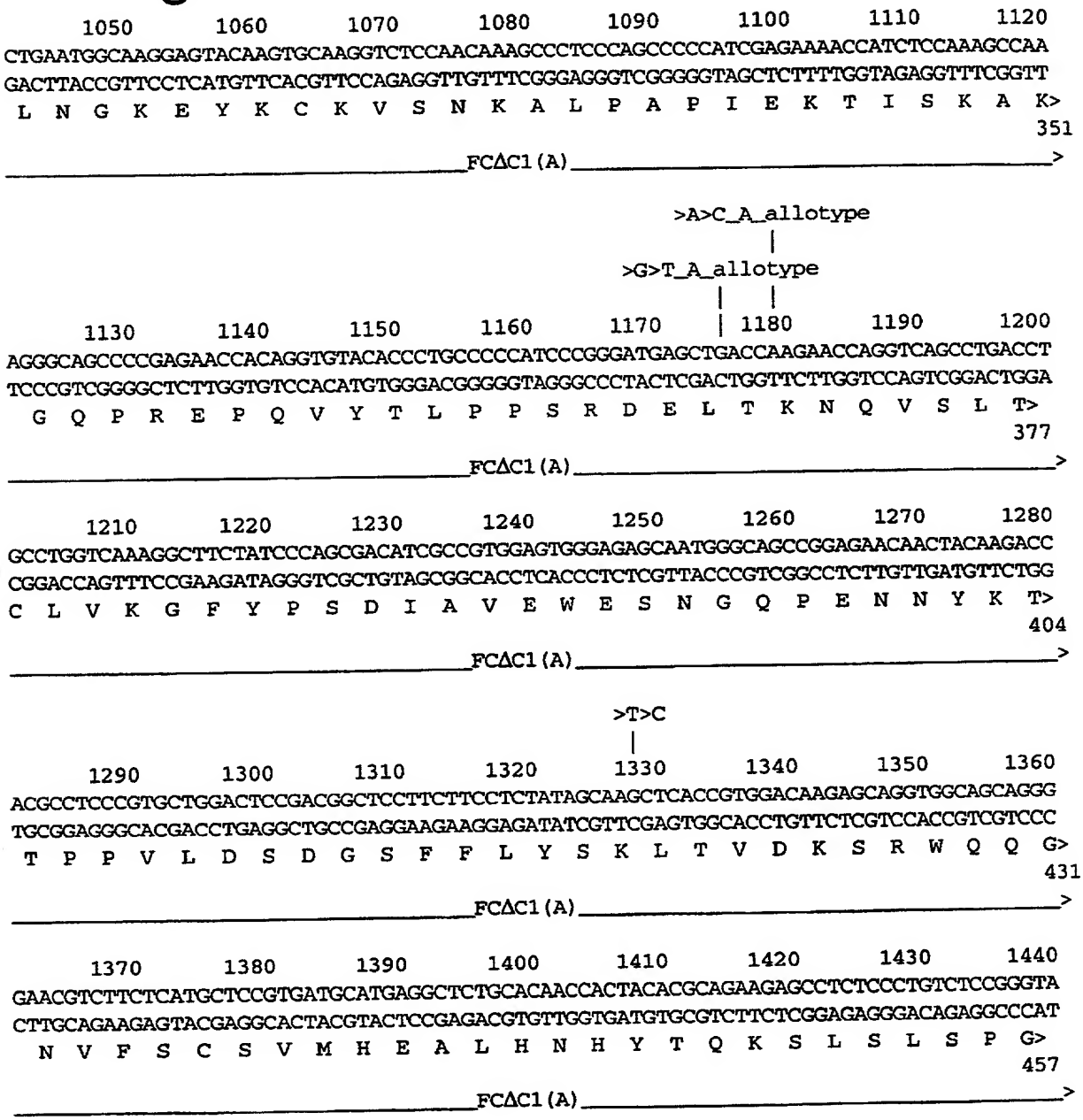
GACAACCTCTTTTCGAACAGAATTTAACATGTCGTTCTTGACTTGATTTACACCCCTAACTGAAGTTGACCCTTATGGGA
S V G E K L V L N C T A R T E L N V G I D F N W E Y P>
164
HFLK1 D3
570 580 590 600 610 620 630 640
TCTTCGAAGCATCAGCATAAGAAACTTGTAAACCGAGACCTAAAAACCCAGTCTGGGAGTGAGATGAAGAAATTTTTGAG
AGAAGCTTCGTAGTCGTATTCTTTGAACATTTGGCTCTGGATTTTTGGGTCAGACCCTCACTCTACTTCTTTAAAAACTC
S S K H Q H K K L V N R D L K T Q S G S E M K K F L S>
191
HFLK1 D3
650 660 670 680 690 700 710 720
CACCTTAACTATAGATGGTGTAACCCGGAGTGACCAAGGATTGTACACCTGTGCAGCATCCAGTGGGCTGATGACCAAGA
GTGGAATTGATATCTACCACATTGGGCCTCACTGGTTCCTAACATGTGGACACGTCGTAGGTCACCCGACTACTGGTTCT
T L T I D G V T R S D Q G L Y T C A A S S G L M T K>
217
HFLK1 D3
>Srf_Bridge_
730 740 750 760 770 780 790 800
AGAACAGCACATTTGTCAGGGTCCATGAAAAGGGCCCGGGCGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAA
TCTTGTCGTGTAAACAGTCCCAGGTACTTTTCCCGGGCCCGCTGTTTTGAGTGTGTACGGGTGGCACGGGTCGTGGACTT
K N S T F V R V H E K>
HFLK1 D3
G P G>
D K T H T C P P C P A P E>
244
FCΔC1(A)
810 820 830 840 850 860 870 880
CTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCAC
GAGGACCCCCCTGGCAGTCAGAAGGAGAAGGGGGGTTTTGGGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTG
L L G G P S V F L F P P K P K D T L M I S R T P E V T>
271
FCΔC1(A)
890 900 910 920 930 940 950 960
ATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATG
TACGCACCACCACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTAC
C V V V D V S H E D P E V K F N W Y V D G V E V H N>
297
FCΔC1(A)
970 980 990 1000 1010 1020 1030 1040
CCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGG
GGTTCTGTTTCGGCGCCCTCCTCGTCATGTTGTCGTGCATGGCACACCAGTCGCAGGAGTGGCAGGACGTGGTCCTGACC
A K T K P R E E Q Y N S T Y R V V S V L T V L H Q D W>
324
FCΔC1(A)

Fig.21C.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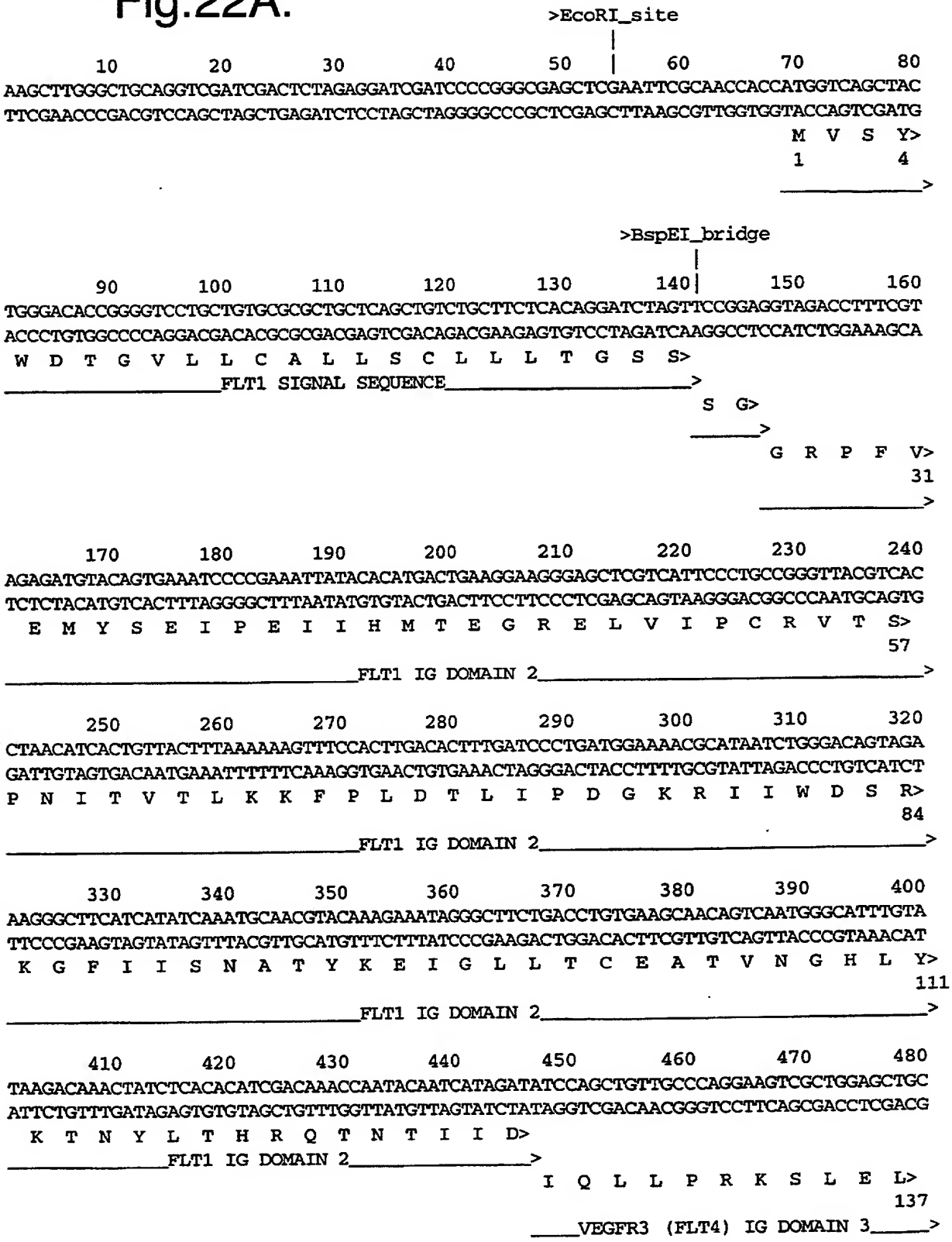
L N G K E Y K C K V S N K A L P A P I E K T I S K A K>
351
FCΔC1(A)
>A>C_A_allotype
>G>T_A_allotype
1130 1140 1150 1160 1170 1180 1190 1200
AGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCT
TCCCGTCGGGGGCTCTTGGTGTCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGA
G Q P R E P Q V Y T L P P S R D E L T K N Q V S L T>
377
FCΔC1(A)
1210 1220 1230 1240 1250 1260 1270 1280
GCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACC
CGGACCAGTTTCCGAAGATAGGGTCGCTGTAGCGGCACCTCACCCTCTCGTTACCCGTCGGCCTCTTGTTGATGTTCTGG
C L V K G F Y P S D I A V E W E S N G Q P E N N Y K T>
404
FCΔC1(A)
>T>C
1290 1300 1310 1320 1330 1340 1350 1360
ACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGG
TGCGGAGGGCACGACCTGAGGCTGCCGAGGAAGAAGGAGATATCGTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTCCC
T P P V L D S D G S F F L Y S K L T V D K S R W Q Q G>
431
FCΔC1(A)
1370 1380 1390 1400 1410 1420 1430 1440
GAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTA
CTTGCAGAAGAGTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCAT
N V F S C S V M H E A L H N H Y T Q K S L S L S P G>
457
FCΔC1(A)
>NotI_site
1450
AATGAGCGGCCGC
TTACTCGCCGGCG
K *>
458

Fig.22A.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M V S Y>
1 4
>BspEI_bridge
90 100 110 120 130 140 150 160
TGGGACACCGGGGTCCTGCTGTGCGCGCTGCTCAGCTGTCTGCTTCTCACAGGATCTAGTTCCGGAGGTAGACCTTTCGT
ACCCTGTGGCCCCAGGACGACACGCGCGACGAGTCGACAGACGAAGAGTGTCCTAGATCAAGGCCTCCATCTGGAAAGCA
W D T G V L L C A L L S C L L L T G S S>
FLT1 SIGNAL SEQUENCE
S G>
G R P F V>
31
170 180 190 200 210 220 230 240
AGAGATGTACAGTGAAATCCCCGAAATTATACACATGACTGAAGGAAGGGAGCTCGTCATTCCCTGCCGGGTTACGTCAC
TCTCTACATGTCACTTTAGGGGCTTTAATATGTGTACTGACTTCCTTCCCTCGAGCAGTAAGGGACGGCCCAATGCAGTG
E M Y S E I P E I I H M T E G R E L V I P C R V T S>
57
FLT1 IG DOMAIN 2
250 260 270 280 290 300 310 320
CTAACATCACTGTTACTTTAAAAAAGTTTCCACTTGACACTTTGATCCCTGATGGAAAACGCATAATCTGGGACAGTAGA
GATTGTAGTGACAATGAAATTTTTTCAAAGGTGAACTGTGAAACTAGGGACTACCTTTTGCGTATTAGACCCTGTCATCT
P N I T V T L K K F P L D T L I P D G K R I I W D S R>
84
FLT1 IG DOMAIN 2
330 340 350 360 370 380 390 400
AAGGGCTTCATCATATCAAATGCAACGTACAAAGAAATAGGGCTTCTGACCTGTGAAGCAACAGTCAATGGGCATTTGTA
TTCCCGAAGTAGTATAGTTTACGTTGCATGTTTCTTTATCCCGAAGACTGGACACTTCGTTGTCAGTTACCCGTAAACAT
K G F I I S N A T Y K E I G L L T C E A T V N G H L Y>
111
FLT1 IG DOMAIN 2
410 420 430 440 450 460 470 480
TAAGACAAACTATCTCACACATCGACAAACCAATACAATCATAGATATCCAGCTGTTGCCCAGGAAGTCGCTGGAGCTGC
ATTCTGTTTGATAGAGTGTGTAGCTGTTTGGTTATGTTAGTATCTATAGGTCGACAACGGGTCCTTCAGCGACCTCGACG
K T N Y L T H R Q T N T I I D>
FLT1 IG DOMAIN 2
I Q L L P R K S L E L>
137
VEGFR3 (FLT4) IG DOMAIN 3

Fig.22B.

```
      490        500        510        520        530        540        550        560
TGGTAGGGGAGAAGCTGGTCCTCAACTGCACCGTGTGGGCTGAGTTTAACTCAGGTGTCACCTTTGACTGGGACTACCCA
ACCATCCCCTCTTCGACCAGGAGTTGACGTGGCACACCCGACTCAAATTGAGTCCACAGTGGAAACTGACCCTGATGGGT
L  V  G  E  K  L  V  L  N  C  T  V  W  A  E  F  N  S  G  V  T  F  D  W  D  Y  P>
                                                                                164
_____________________________VEGFR3 (FLT4) IG DOMAIN 3_____________________________>

      570        580        590        600        610        620        630        640
GGGAAGCAGGCAGAGCGGGGTAAGTGGGTGCCCGAGCGACGCTCCCAACAGACCCACACAGAACTCTCCAGCATCCTGAC
CCCTTCGTCCGTCTCGCCCCATTCACCCACGGGCTCGCTGCGAGGGTTGTCTGGGTGTGTCTTGAGAGGTCGTAGGACTG
 G  K  Q  A  E  R  G  K  W  V  P  E  R  R  S  Q  Q  T  H  T  E  L  S  S  I  L  T>
                                                                                191
_____________________________VEGFR3 (FLT4) IG DOMAIN 3_____________________________>

      650        660        670        680        690        700        710        720
CATCCACAACGTCAGCCAGCACGACCTGGGCTCGTATGTGTGCAAGGCCAACAACGGCATCCAGCGATTTCGGGAGAGCA
GTAGGTGTTGCAGTCGGTCGTGCTGGACCCGAGCATACACACGTTCCGGTTGTTGCCGTAGGTCGCTAAAGCCCTCTCGT
  I  H  N  V  S  Q  H  D  L  G  S  Y  V  C  K  A  N  N  G  I  Q  R  F  R  E  S>
                                                                                217
_____________________________VEGFR3 (FLT4) IG DOMAIN 3_____________________________>

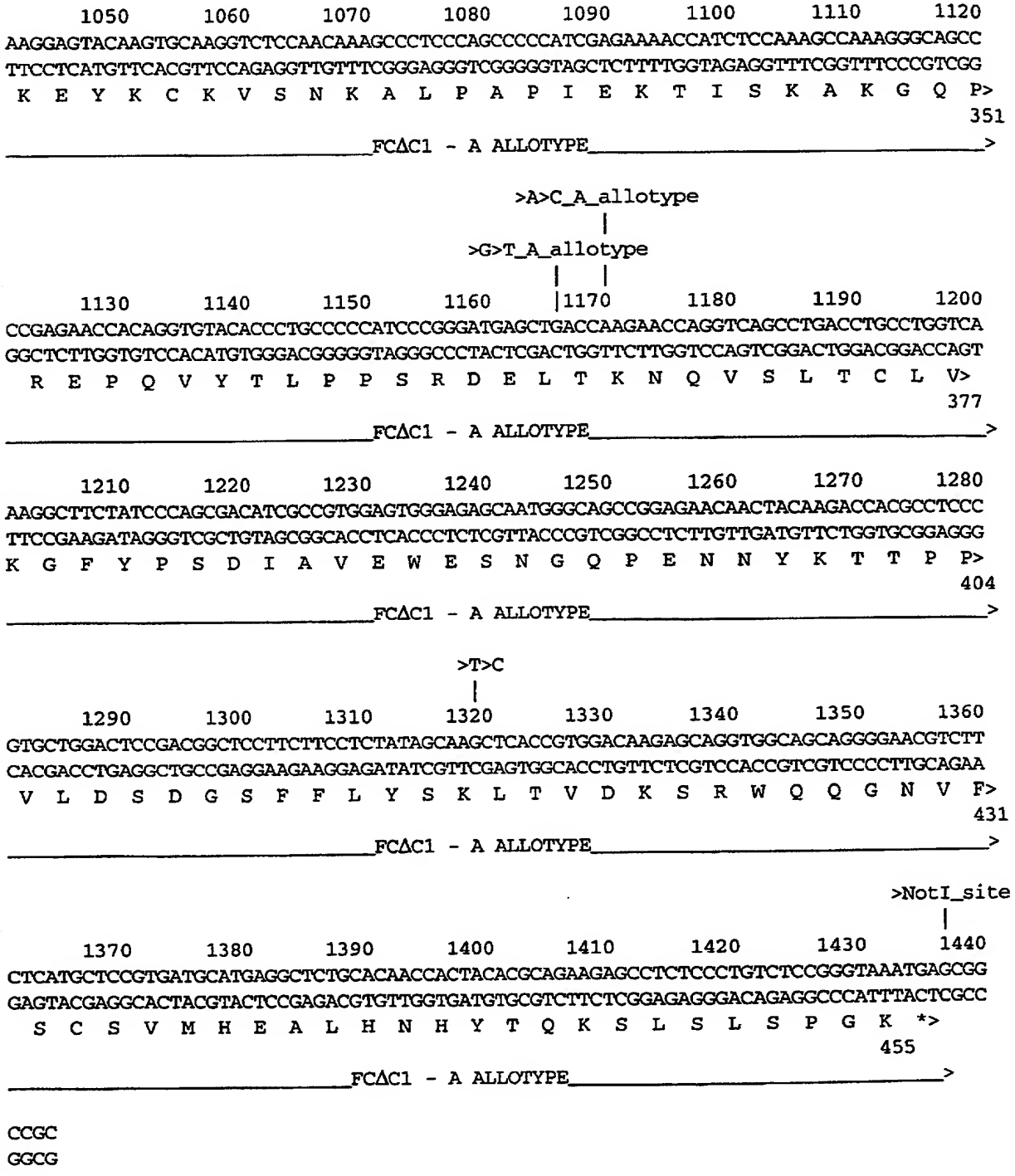
      730        740        750        760        770        780        790        800
CCGAGGTCATTGTGCATGAAAATGGCCCGGGCGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGGGG
GGCTCCAGTAACACGTACTTTTACCGGGCCCGCTGTTTTGAGTGTGTACGGGTGGCACGGGTCGTGGACTTGAGGACCCC
T  E  V  I  V  H  E  N>
___VEGFR3 (FLT4) IG____>
                     G  P  G>
                     _________>
                               D  K  T  H  T  C  P  P  C  P  A  P  E  L  L  G>
                                                                             244
                               _______________FCΔC1 - A ALLOTYPE_______________>

      810        820        830        840        850        860        870        880
GGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGTCACATGCGTGGT
CCTGGCAGTCAGAAGGAGAAGGGGGGTTTTGGGTTCCTGTGGGAGTACTAGAGGGCCTGGGGACTCCAGTGTACGCACCA
 G  P  S  V  F  L  F  P  P  K  P  K  D  T  L  M  I  S  R  T  P  E  V  T  C  V  V>
                                                                                271
_____________________________FCΔC1 - A ALLOTYPE_____________________________>

      890        900        910        920        930        940        950        960
GGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAA
CCACCTGCACTCGGTGCTTCTGGGACTCCAGTTCAAGTTGACCATGCACCTGCCGCACCTCCACGTATTACGGTTCTGTT
  V  D  V  S  H  E  D  P  E  V  K  F  N  W  Y  V  D  G  V  E  V  H  N  A  K  T>
                                                                                297
_____________________________FCΔC1 - A ALLOTYPE_____________________________>

      970        980        990       1000       1010       1020       1030       1040
AGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGC
TCGGCGCCCTCCTCGTCATGTTGTCGTGCATGGCACACCAGTCGCAGGAGTGGCAGGACGTGGTCCTGACCGACTTACCG
K  P  R  E  E  Q  Y  N  S  T  Y  R  V  V  S  V  L  T  V  L  H  Q  D  W  L  N  G>
                                                                                324
_____________________________FCΔC1 - A ALLOTYPE_____________________________>
```

Fig.22C.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K E Y K C K V S N K A L P A P I E K T I S K A K G Q P>
351
FCΔC1 - A ALLOTYPE
>A>C_A_allotype
>G>T_A_allotype
1130 1140 1150 1160 1170 1180 1190 1200
CCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCA
GGCTCTTGGTGTCCACATGTGGGACGGGGGTAGGGCCCTACTCGACTGGTTCTTGGTCCAGTCGGACTGGACGGACCAGT
R E P Q V Y T L P P S R D E L T K N Q V S L T C L V>
377
FCΔC1 - A ALLOTYPE
1210 1220 1230 1240 1250 1260 1270 1280
AAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACGCCTCCC
TTCCGAAGATAGGGTCGCTGTAGCGGCACCTCACCCTCTCGTTACCCGTCGGCCTCTTGTTGATGTTCTGGTGCGGAGGG
K G F Y P S D I A V E W E S N G Q P E N N Y K T T P P>
404
FCΔC1 - A ALLOTYPE
>T>C
1290 1300 1310 1320 1330 1340 1350 1360
GTGCTGGACTCCGACGGCTCCTTCTTCCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTT
CACGACCTGAGGCTGCCGAGGAAGAAGGAGATATCGTTCGAGTGGCACCTGTTCTCGTCCACCGTCGTCCCCTTGCAGAA
V L D S D G S F F L Y S K L T V D K S R W Q Q G N V F>
431
FCΔC1 - A ALLOTYPE
>NotI_site
1370 1380 1390 1400 1410 1420 1430 1440
CTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGAGCGG
GAGTACGAGGCACTACGTACTCCGAGACGTGTTGGTGATGTGCGTCTTCTCGGAGAGGGACAGAGGCCCATTTACTCGCC
S C S V M H E A L H N H Y T Q K S L S L S P G K *>
455
FCΔC1 - A ALLOTYPE
CCGC
GGCG

Fig.23.

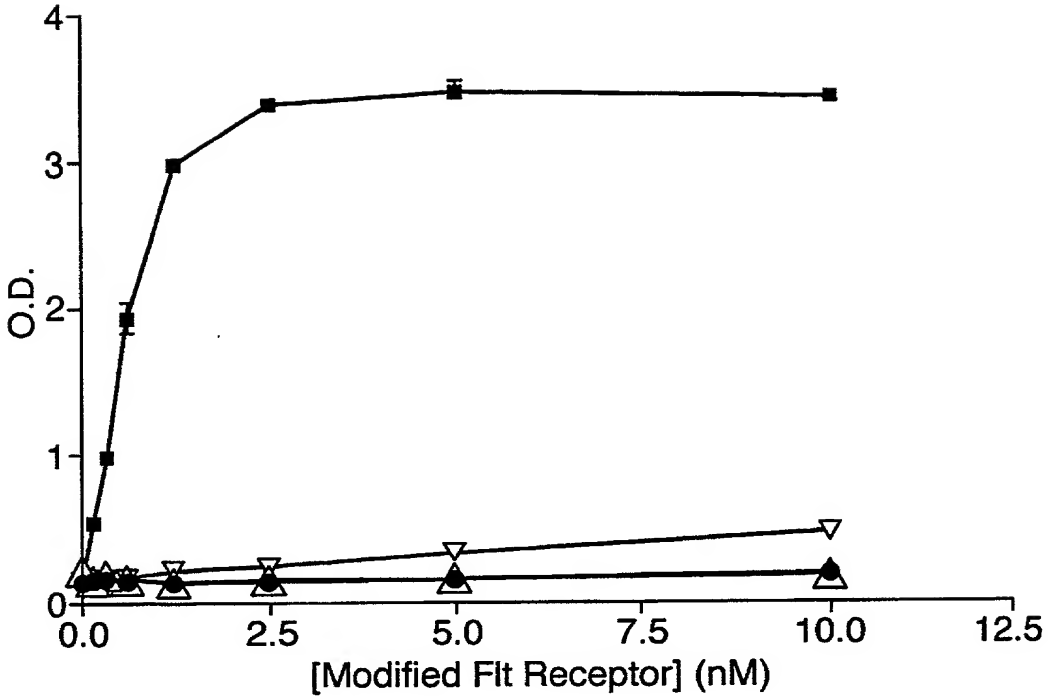
4
3
2
1
0
O.D.
0.0
2.5
5.0
7.5
10.0
12.5
[Modified Flt Receptor] (nM)
● Flt1D2Flk1D3.FcdeltaC1(a)
△ Flt1D2VEGFR3D3.FcdeltaC1(a)
▽ TIE2-Fc
■ Flt1(1-3)-Fc

Fig.24A.

```
          10          20          30          40          50          60
           *           *           *           *           *           *
ATG GTC AGC TAC TGG GAC ACC GGG GTC CTG CTG TGC GCG CTG CTC AGC TGT CTG CTT CTC
TAC CAG TCG ATG ACC CTG TGG CCC CAG GAC GAC ACG CGC GAC GAG TCG ACA GAC GAA GAG
 M   V   S   Y   W   D   T   G   V   L   L   C   A   L   L   S   C   L   L   L>
_1_____________5_________hFLT1 SIGNAL SEQUENCE_______15_____________________20>

          70          80          90          100         110         120
           *           *           *           *           *           *
ACA GGA TCT AGT TCC GGA AGT GAT ACC GGT AGA CCT TTC GTA GAG ATG TAC AGT GAA ATC
TGT CCT AGA TCA AGG CCT TCA CTA TGG CCA TCT GGA AAG CAT CTC TAC ATG TCA CTT TAG
 T   G   S   S   S   G>
21_hFLT1 SIGNAL SEQ_26>
                         S   D   T   G   R   P   F   V   E   M   Y   S   E   I>
                        _27_____30___hFLT1 IG DOMAIN 2_____________________40>

          130         140         150         160         170         180
           *           *           *           *           *           *
CCC GAA ATT ATA CAC ATG ACT GAA GGA AGG GAG CTC GTC ATT CCC TGC CGG GTT ACG TCA
GGG CTT TAA TAT GTG TAC TGA CTT CCT TCC CTC GAG CAG TAA GGG ACG GCC CAA TGC AGT
 P   E   I   I   H   M   T   E   G   R   E   L   V   I   P   C   R   V   T   S>
41_____________45_____________hFLT1 IG DOMAIN 2_________55_____________________60>

          190         200         210         220         230         240
           *           *           *           *           *           *
CCT AAC ATC ACT GTT ACT TTA AAA AAG TTT CCA CTT GAC ACT TTG ATC CCT GAT GGA AAA
GGA TTG TAG TGA CAA TGA AAT TTT TTC AAA GGT GAA CTG TGA AAC TAG GGA CTA CCT TTT
 P   N   I   T   V   T   L   K   K   F   P   L   D   T   L   I   P   D   G   K>
61_____________65_____________hFLT1 IG DOMAIN 2_________75_____________________80>

          250         260         270         280         290         300
           *           *           *           *           *           *
CGC ATA ATC TGG GAC AGT AGA AAG GGC TTC ATC ATA TCA AAT GCA ACG TAC AAA GAA ATA
GCG TAT TAG ACC CTG TCA TCT TTC CCG AAG TAG TAT AGT TTA CGT TGC ATG TTT CTT TAT
 R   I   I   W   D   S   R   K   G   F   I   I   S   N   A   T   Y   K   E   I>
81_____________85_____________hFLT1 IG DOMAIN 2_________95_____________________100>

          310         320         330         340         350         360
           *           *           *           *           *           *
GGG CTT CTG ACC TGT GAA GCA ACA GTC AAT GGG CAT TTG TAT AAG ACA AAC TAT CTC ACA
CCC GAA GAC TGG ACA CTT CGT TGT CAG TTA CCC GTA AAC ATA TTC TGT TTG ATA GAG TGT
 G   L   L   T   C   E   A   T   V   N   G   H   L   Y   K   T   N   Y   L   T>
101____________105____________hFLT1 IG DOMAIN 2_________115____________________120>

          370         380         390         400         410         420
           *           *           *           *           *           *
CAT CGA CAA ACC AAT ACA ATC ATA GAT GTG GTT CTG AGT CCG TCT CAT GGA ATT GAA CTA
GTA GCT GTT TGG TTA TGT TAG TAT CTA CAC CAA GAC TCA GGC AGA GTA CCT TAA CTT GAT
 H   R   Q   T   N   T   I   I   D>
121_____hFLT1 IG DOMAIN 2_____129_>
                                    V   V   L   S   P   S   H   G   I   E   L>
                                   130_____hFLK1 IG DOMAIN 3_____________140>
```

Fig.24B.

```
          430         440         450         460         470         480
           *           *           *           *           *           *
TCT GTT GGA GAA AAG CTT GTC TTA AAT TGT ACA GCA AGA ACT GAA CTA AAT GTG GGG ATT
AGA CAA CCT CTT TTC GAA CAG AAT TTA ACA TGT CGT TCT TGA CTT GAT TTA CAC CCC TAA
 S   V   G   E   K   L   V   L   N   C   T   A   R   T   E   L   N   V   G   I>
141_____________145_____________hFLK1 IG DOMAIN 3_____155_____________160>

          490         500         510         520         530         540
           *           *           *           *           *           *
GAC TTC AAC TGG GAA TAC CCT TCT TCG AAG CAT CAG CAT AAG AAA CTT GTA AAC CGA GAC
CTG AAG TTG ACC CTT ATG GGA AGA AGC TTC GTA GTC GTA TTC TTT GAA CAT TTG GCT CTG
 D   F   N   W   E   Y   P   S   S   K   H   Q   H   K   K   L   V   N   R   D>
161_____________165_____________hFLK1 IG DOMAIN 3_____175_____________180>

          550         560         570         580         590         600
           *           *           *           *           *           *
CTA AAA ACC CAG TCT GGG AGT GAG ATG AAG AAA TTT TTG AGC ACC TTA ACT ATA GAT GGT
GAT TTT TGG GTC AGA CCC TCA CTC TAC TTC TTT AAA AAC TCG TGG AAT TGA TAT CTA CCA
 L   K   T   Q   S   G   S   E   M   K   K   F   L   S   T   L   T   I   D   G>
181_____________185_____________hFLK1 IG DOMAIN 3_____195_____________200>

          610         620         630         640         650         660
           *           *           *           *           *           *
GTA ACC CGG AGT GAC CAA GGA TTG TAC ACC TGT GCA GCA TCC AGT GGG CTG ATG ACC AAG
CAT TGG GCC TCA CTG GTT CCT AAC ATG TGG ACA CGT CGT AGG TCA CCC GAC TAC TGG TTC
 V   T   R   S   D   Q   G   L   Y   T   C   A   A   S   S   G   L   M   T   K>
201_____________205_____________hFLK1 IG DOMAIN 3_____215_____________220>

          670         680         690         700         710         720
           *           *           *           *           *           *
AAG AAC AGC ACA TTT GTC AGG GTC CAT GAA AAG GAC AAA ACT CAC ACA TGC CCA CCG TGC
TTC TTG TCG TGT AAA CAG TCC CAG GTA CTT TTC CTG TTT TGA GTG TGT ACG GGT GGC ACG
 K   N   S   T   F   V   R   V   H   E   K>
221_____hFLK1 IG DOMAIN 3_____231>
                                            D   K   T   H   T   C   P   P   C>
                                        232_____hFCAC1 A _____240>

          730         740         750         760         770         780
           *           *           *           *           *           *
CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC TTC CCC CCA AAA CCC AAG GAC
GGT CGT GGA CTT GAG GAC CCC CCT GGC AGT CAG AAG GAG AAG GGG GGT TTT GGG TTC CTG
 P   A   P   E   L   L   G   G   P   S   V   F   L   F   P   P   K   P   K   D>
241_____________245_____hFCAC1 A _____255_____________260>

          790         800         810         820         830         840
           *           *           *           *           *           *
ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA TGC GTG GTG GTG GAC GTG AGC CAC GAA
TGG GAG TAC TAG AGG GCC TGG GGA CTC CAG TGT ACG CAC CAC CAC CTG CAC TCG GTG CTT
 T   L   M   I   S   R   T   P   E   V   T   C   V   V   V   D   V   S   H   E>
261_____________265_____hFCAC1 A _____275_____________280>

          850         860         870         880         890         900
           *           *           *           *           *           *
GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA
CTG GGA CTC CAG TTC AAG TTG ACC ATG CAC CTG CCG CAC CTC CAC GTA TTA CGG TTC TGT
 D   P   E   V   K   F   N   W   Y   V   D   G   V   E   V   H   N   A   K   T>
281_____________285_____hFCAC1 A _____295_____________300>
```

Fig.24C.

```
          910         920         930         940         950         960
            *           *           *           *           *           *
AAG CCG CGG GAG GAG CAG TAC AAC AGC ACG TAC CGT GTG GTC AGC GTC CTC ACC GTC CTG
TTC GGC GCC CTC CTC GTC ATG TTG TCG TGC ATG GCA CAC CAG TCG CAG GAG TGG CAG GAC
 K   P   R   E   E   Q   Y   N   S   T   Y   R   V   V   S   V   L   T   V   L>
301_____________305_____________hFCΔC1 A _____________315_____________320>

          970         980         990        1000        1010        1020
            *           *           *           *           *           *
CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC AAC AAA GCC CTC CCA
GTG GTC CTG ACC GAC TTA CCG TTC CTC ATG TTC ACG TTC CAG AGG TTG TTT CGG GAG GGT
 H   Q   D   W   L   N   G   K   E   Y   K   C   K   V   S   N   K   A   L   P>
321_____________325_____________hFCΔC1 A _____________335_____________340>

          1030        1040        1050        1060        1070        1080
            *           *           *           *           *           *
GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC
CGG GGG TAG CTC TTT TGG TAG AGG TTT CGG TTT CCC GTC GGG GCT CTT GGT GTC CAC ATG
 A   P   I   E   K   T   I   S   K   A   K   G   Q   P   R   E   P   Q   V   Y>
341_____________345_____________hFCΔC1 A _____________355_____________360>

          1090        1100        1110        1120        1130        1140
            *           *           *           *           *           *
ACC CTG CCC CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC
TGG GAC GGG GGT AGG GCC CTA CTC GAC TGG TTC TTG GTC CAG TCG GAC TGG ACG GAC CAG
 T   L   P   P   S   R   D   E   L   T   K   N   Q   V   S   L   T   C   L   V>
361_____________365_____________hFCΔC1 A _____________375_____________380>

          1150        1160        1170        1180        1190        1200
            *           *           *           *           *           *
AAA GGC TTC TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG AAC
TTT CCG AAG ATA GGG TCG CTG TAG CGG CAC CTC ACC CTC TCG TTA CCC GTC GGC CTC TTG
 K   G   F   Y   P   S   D   I   A   V   E   W   E   S   N   G   Q   P   E   N>
381_____________385_____________hFCΔC1 A _____________395_____________400>

          1210        1220        1230        1240        1250        1260
            *           *           *           *           *           *
AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC TTC CTC TAC AGC AAG
TTG ATG TTC TGG TGC GGA GGG CAC GAC CTG AGG CTG CCG AGG AAG AAG GAG ATG TCG TTC
 N   Y   K   T   T   P   P   V   L   D   S   D   G   S   F   F   L   Y   S   K>
401_____________405_____________hFCΔC1 A _____________415_____________420>

          1270        1280        1290        1300        1310        1320
            *           *           *           *           *           *
CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG GGG AAC GTC TTC TCA TGC TCC GTG ATG CAT
GAG TGG CAC CTG TTC TCG TCC ACC GTC GTC CCC TTG CAG AAG AGT ACG AGG CAC TAC GTA
 L   T   V   D   K   S   R   W   Q   Q   G   N   V   F   S   C   S   V   M   H>
421_____________425_____________hFCΔC1 A _____________435_____________440>

          1330        1340        1350        1360        1370
            *           *           *           *           *
GAG GCT CTG CAC AAC CAC TAC ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGA
CTC CGA GAC GTG TTG GTG ATG TGC GTC TTC TCG GAG AGG GAC AGA GGC CCA TTT ACT
 E   A   L   H   N   H   Y   T   Q   K   S   L   S   L   S   P   G   K   *>
441_____________445_____________hFCΔC1 A _____________455_____458____>
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Fig.25A.

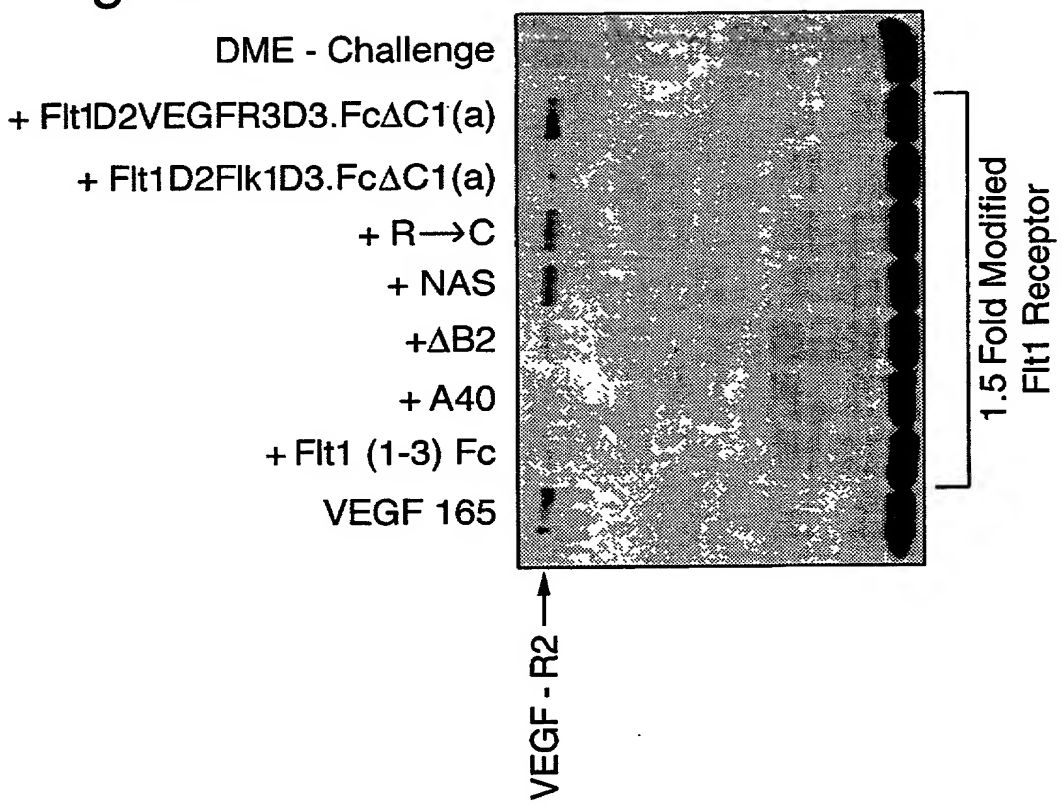


Fig.25B.

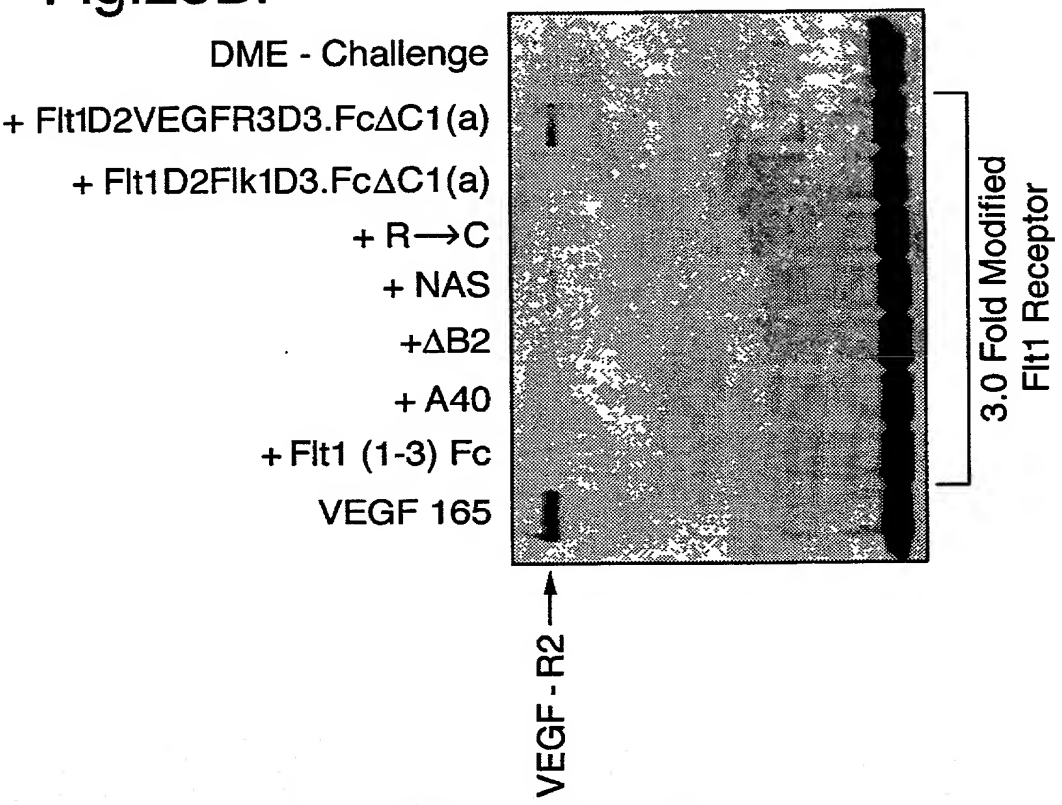


Fig.25C.

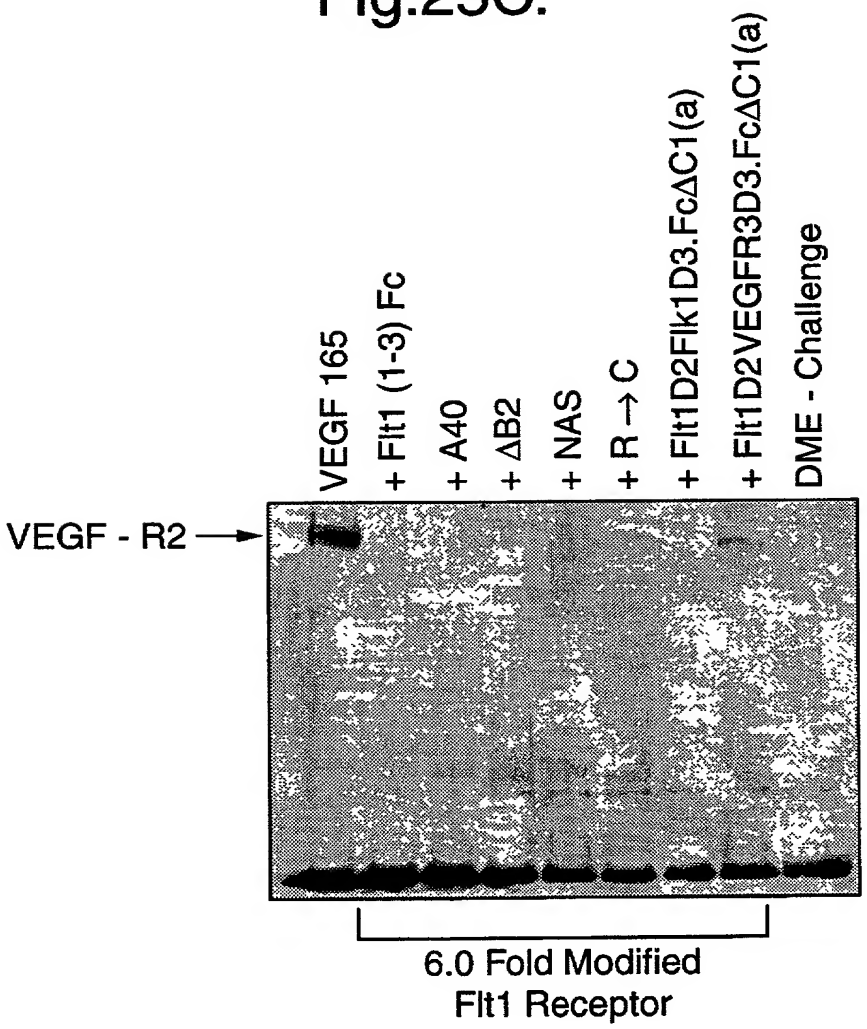

VEGF 165
+ Flt1 (1-3) Fc
+ A40
+ ΔB2
+ NAS
+ R → C
+ Flt1D2Flk1D3.FcΔC1(a)
+ Flt1D2VEGFR3D3.FcΔC1(a)
DME - Challenge
VEGF - R2
6.0 Fold Modified
Flt1 Receptor

Fig.26A.

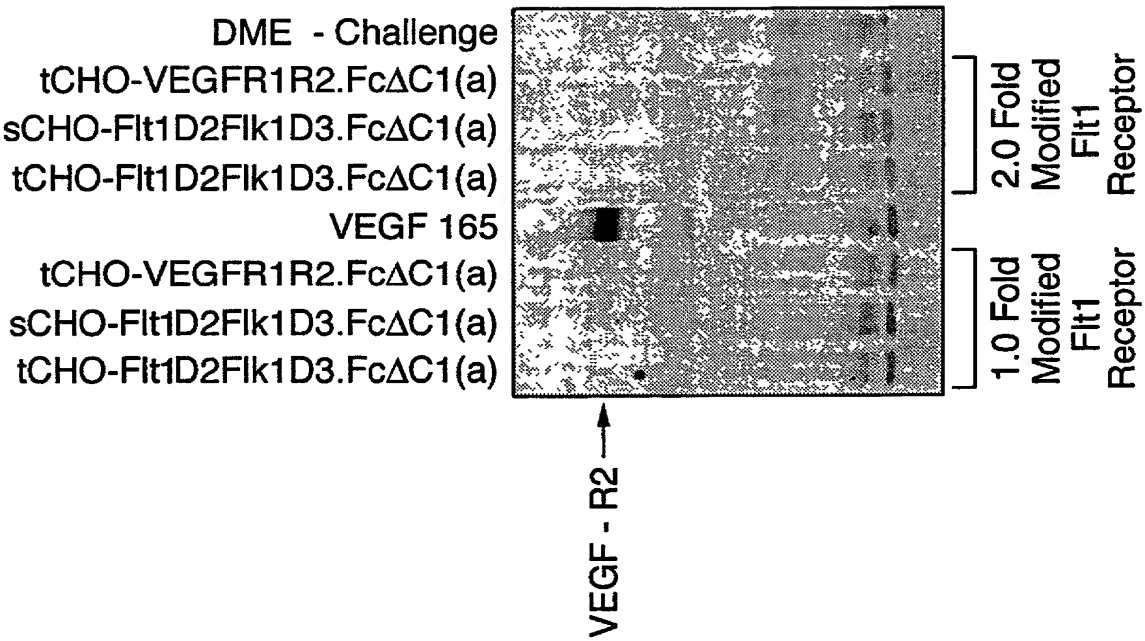


Fig.26B.

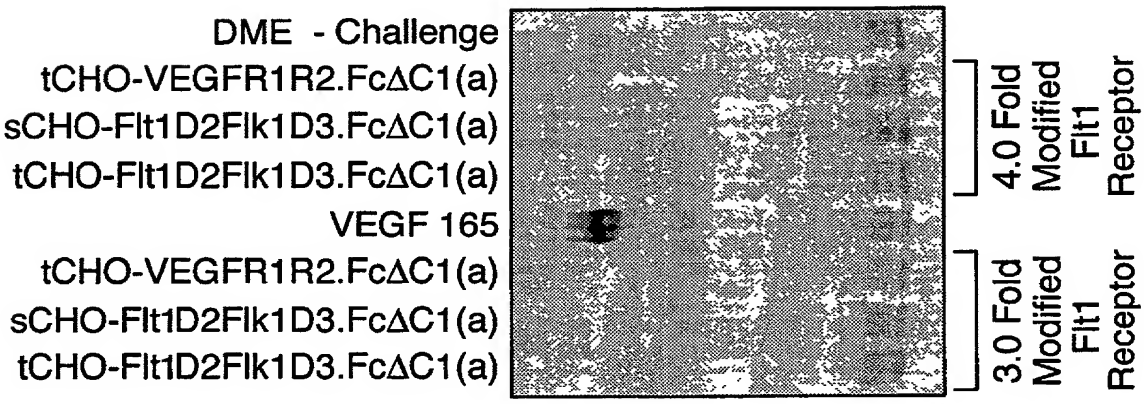


Fig.27.

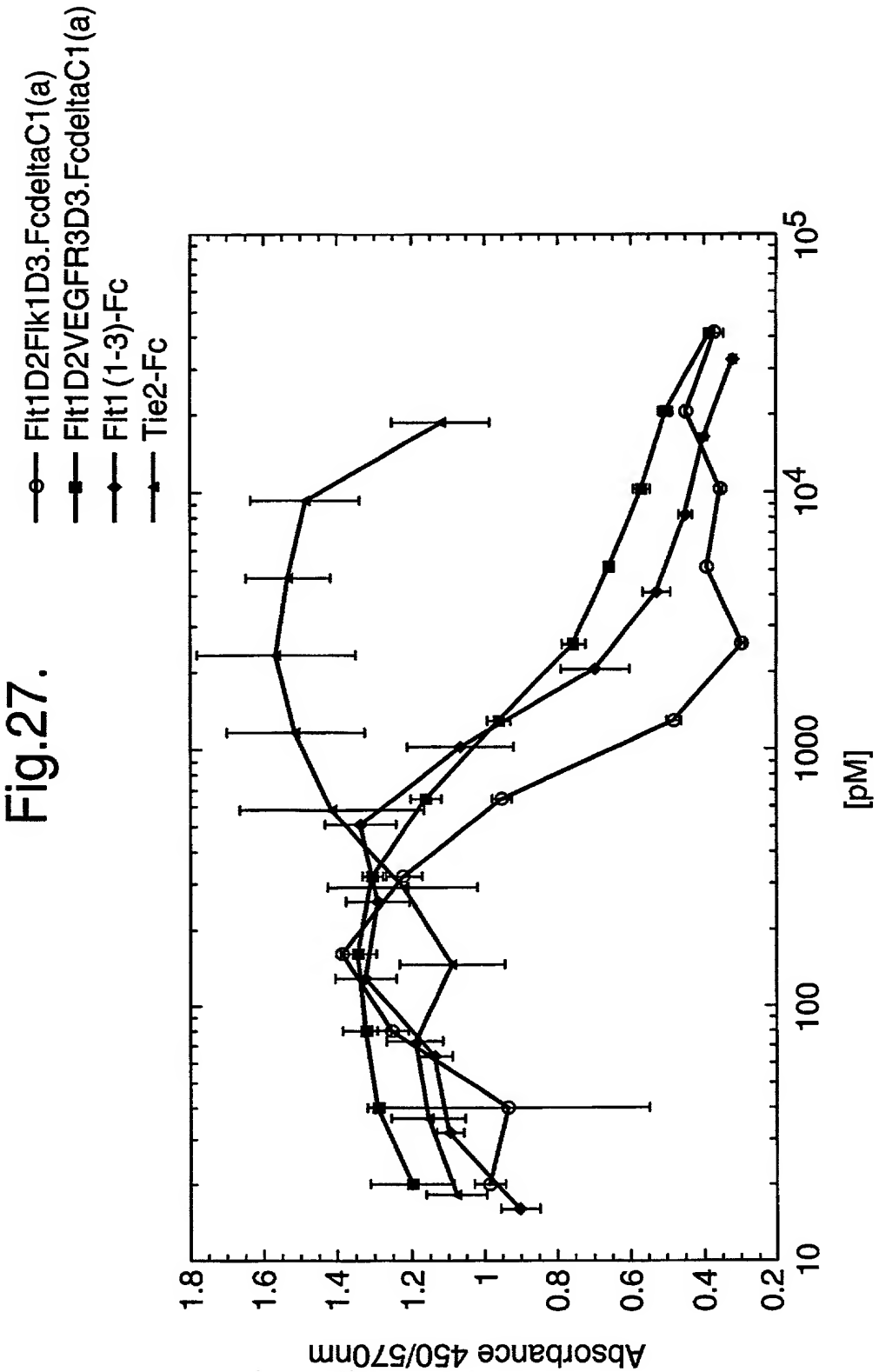

Flt1D2Flk1D3.FcdeltaC1(a)
Flt1D2VEGFR3D3.FcdeltaC1(a)
Flt1(1-3)-Fc
Tie2-Fc
Absorbance 450/570nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
10
100
1000
10^4
10^5
[pM]

Fig.28.

Binding Stoichiometry of hVEGF165 to Flt1D2Flk1D3.FcΔC1(a) & VEGFR1R2-FcΔC1(a)		
hVEGF165 (nM)	VEGF/Flt1D2Flk1D3.FcΔC1(a)	VEGF/VEGFR1R2-FcΔC1(a)
1	0.93	0.98
10	0.97	0.94
50	1	0.99
Average ± StDev	0.96 ± 0.03	0.97 ± 0.02

Fig.29.

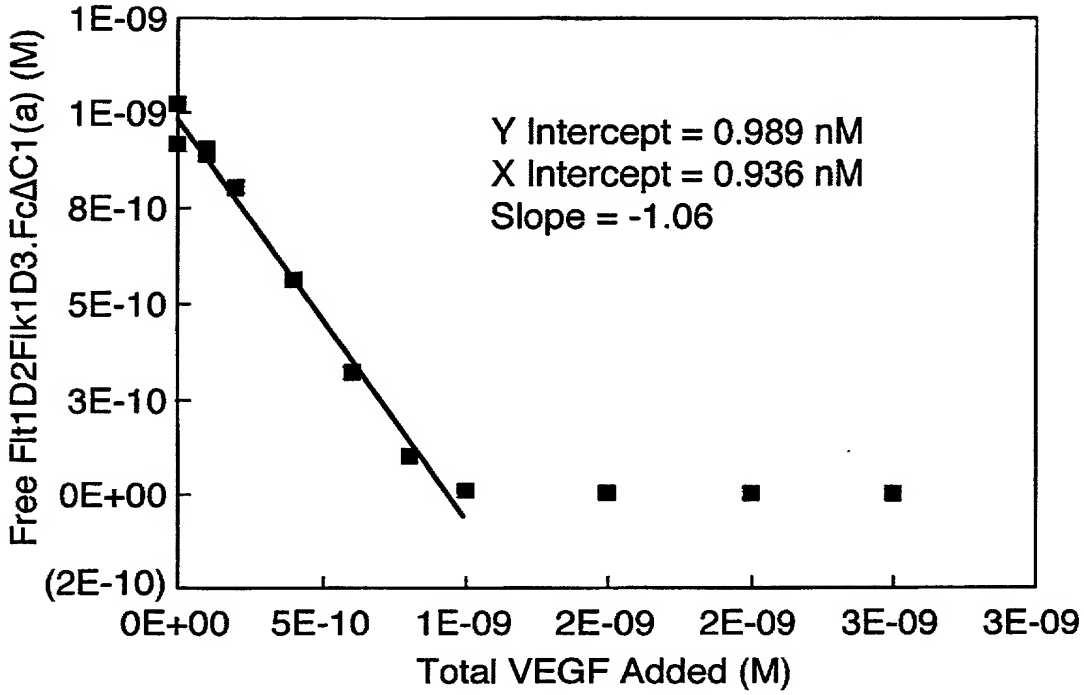

Free Flt1D2Flk1D3.FcΔC1(a) (M)
1E-09
1E-09
8E-10
5E-10
3E-10
0E+00
(2E-10)
Y Intercept = 0.989 nM
X Intercept = 0.936 nM
Slope = -1.06
0E+00
5E-10
1E-09
2E-09
2E-09
3E-09
3E-09
Total VEGF Added (M)

Fig.30.

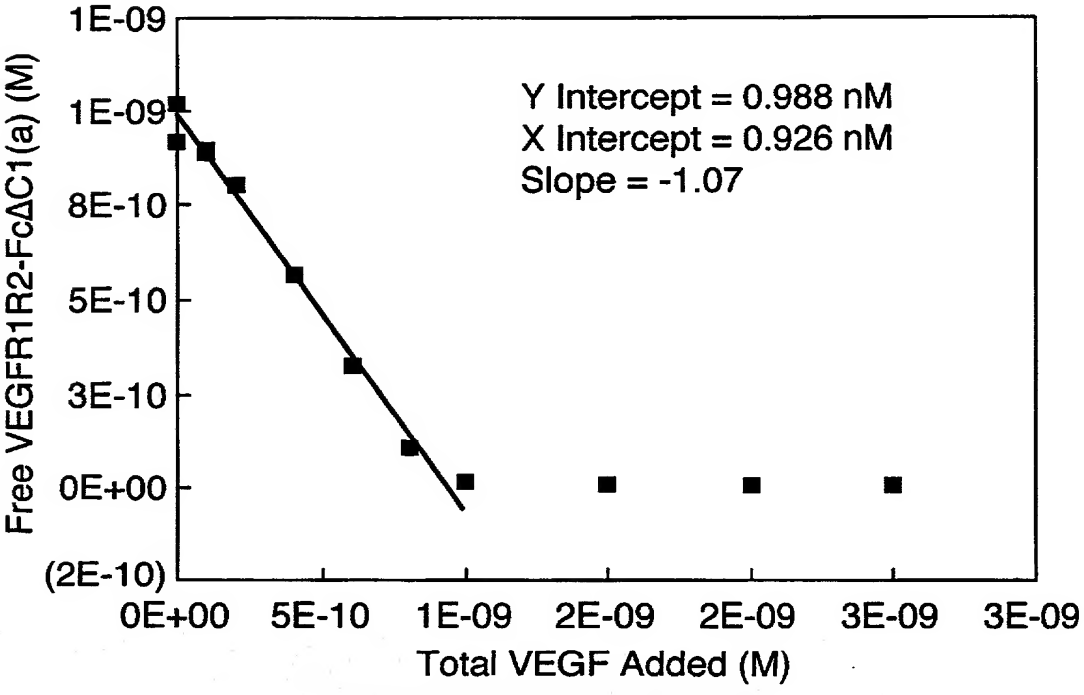

Free VEGFR1R2-FcΔC1(a) (M)
1E-09
1E-09
8E-10
5E-10
3E-10
0E+00
(2E-10)
Y Intercept = 0.988 nM
X Intercept = 0.926 nM
Slope = -1.07
0E+00
5E-10
1E-09
2E-09
2E-09
3E-09
3E-09
Total VEGF Added (M)

Fig.31.

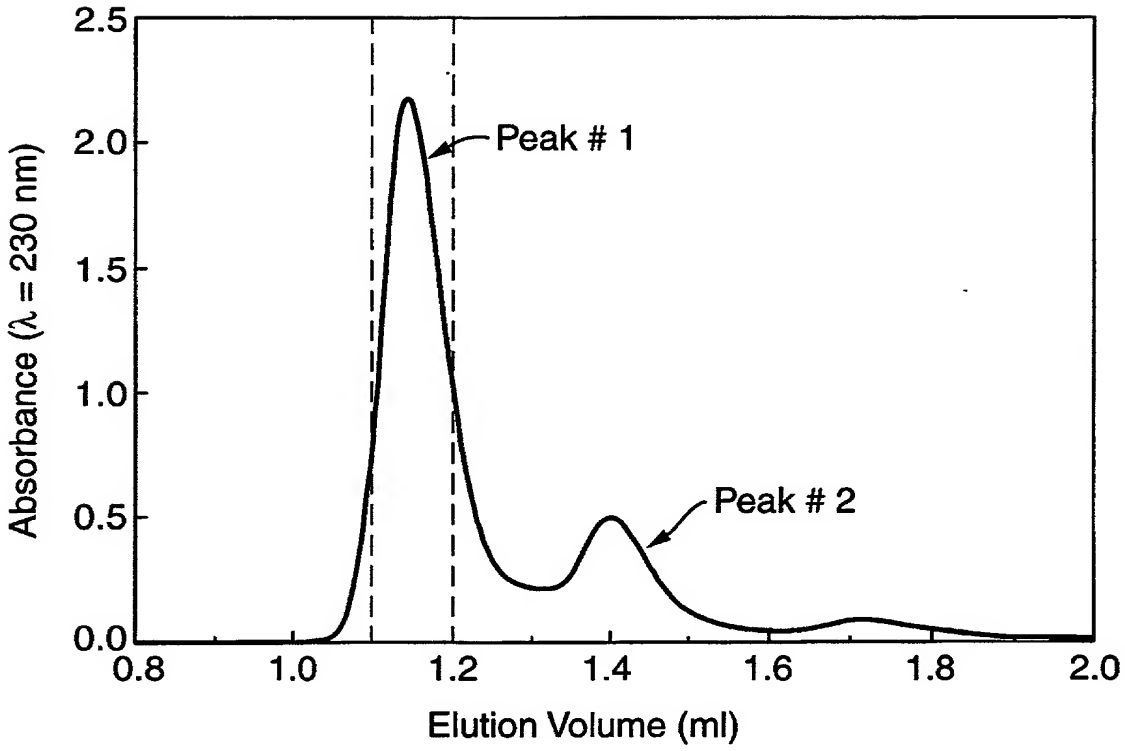

2.5
2.0
1.5
1.0
0.5
0.0
Absorbance (λ = 230 nm)
Peak # 1
Peak # 2
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Elution Volume (ml)

Fig.32.

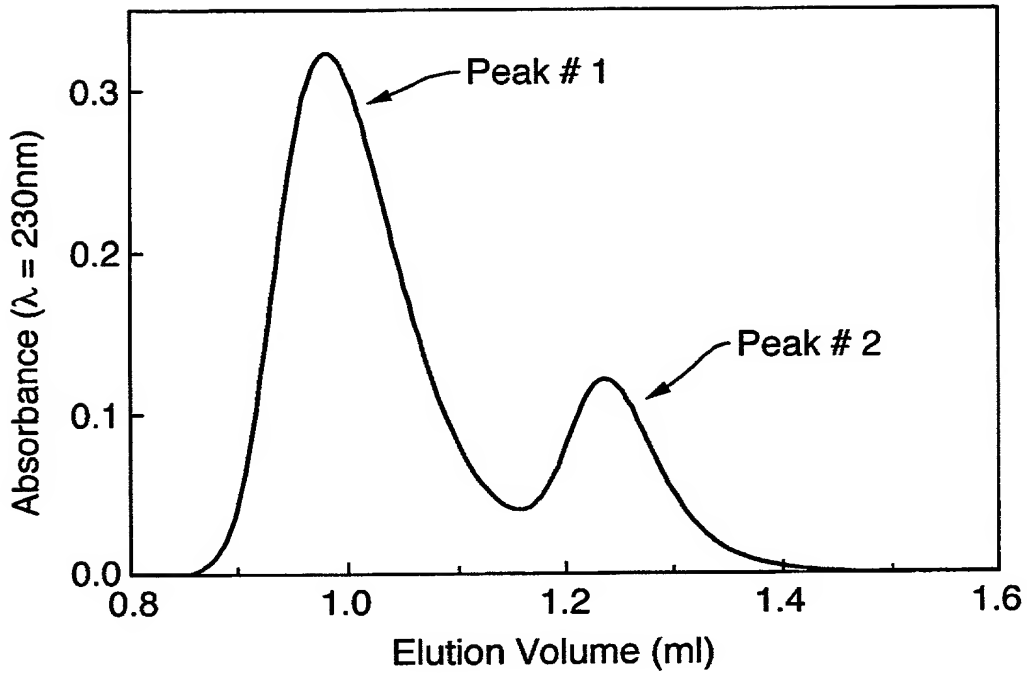

Peak # 1
Peak # 2
Absorbance (λ = 230nm)
0.0
0.1
0.2
0.3
0.8
1.0
1.2
1.4
1.6
Elution Volume (ml)

Fig.33.

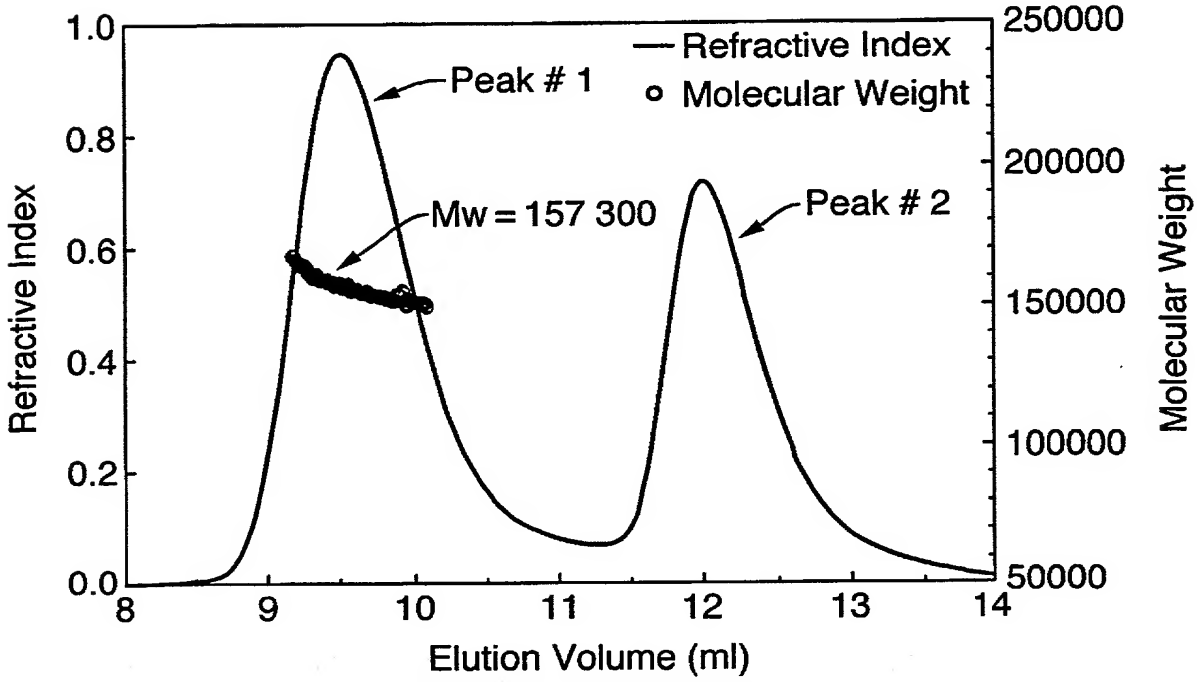

Refractive Index
Molecular Weight
Peak # 1
Peak # 2
Mw = 157 300
Refractive Index
Molecular Weight
0.0
0.2
0.4
0.6
0.8
1.0
50000
100000
150000
200000
250000
8
9
10
11
12
13
14
Elution Volume (ml)

Fig.34.

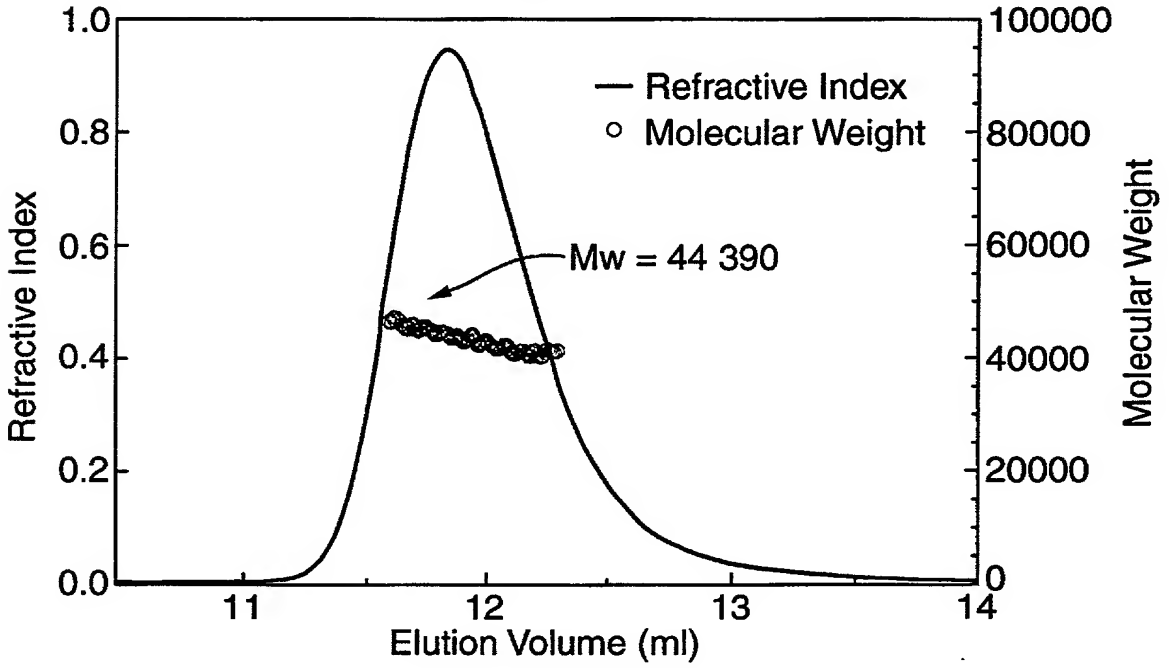

1.0
0.8
0.6
0.4
0.2
0.0
Refractive Index
100000
80000
60000
40000
20000
0
Molecular Weight
— Refractive Index
○ Molecular Weight
Mw = 44 390
11
12
13
14
Elution Volume (ml)

Fig.35.

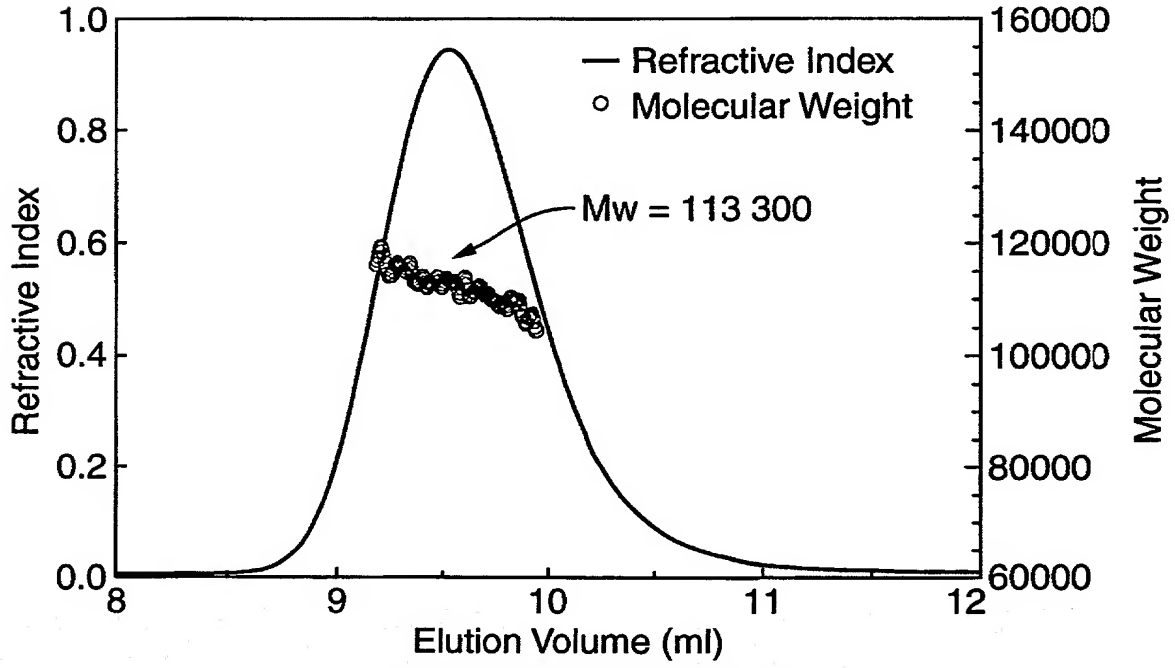

1.0
0.8
0.6
0.4
0.2
0.0
Refractive Index
160000
140000
120000
100000
80000
60000
Molecular Weight
— Refractive Index
○ Molecular Weight
Mw = 113 300
8
9
10
11
12
Elution Volume (ml)

Fig.36.

50
GRPFVEMYSEIPEIIHMTEGRELVIPCRVTSPNITVTLKKFPLDTLIPDG
100
KRIIWDSRKGFIISNATYKEIGLLTCEATVNGHLYKTNYLTHRQTNTIID
150
VVLSPSHGIELSVGEKLVLNCTARTELNVGIDFNWEYPSSKHQHKKLVNR
200
DLKTQSGSEMKKFLSTLTIDGVTRSDQGLYTCAASSGLMTKKNSTFVRVH
250
EKGPGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD
300
VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
350
GKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSL
400
TCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Fig.37.

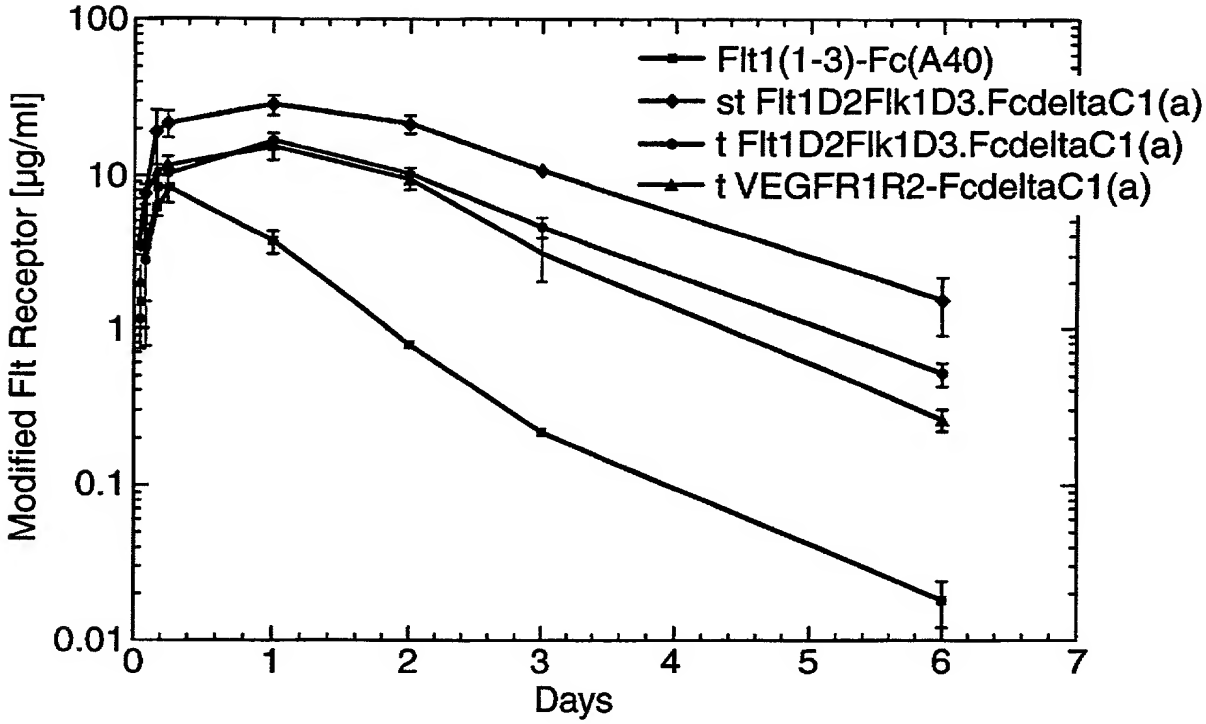

Flt1(1-3)-Fc(A40)
st Flt1D2Flk1D3.FcdeltaC1(a)
t Flt1D2Flk1D3.FcdeltaC1(a)
t VEGFR1R2-FcdeltaC1(a)
Modified Flt Receptor [µg/ml]
100
10
1
0.1
0.01
0
1
2
3
4
5
6
7
Days

Fig.38.

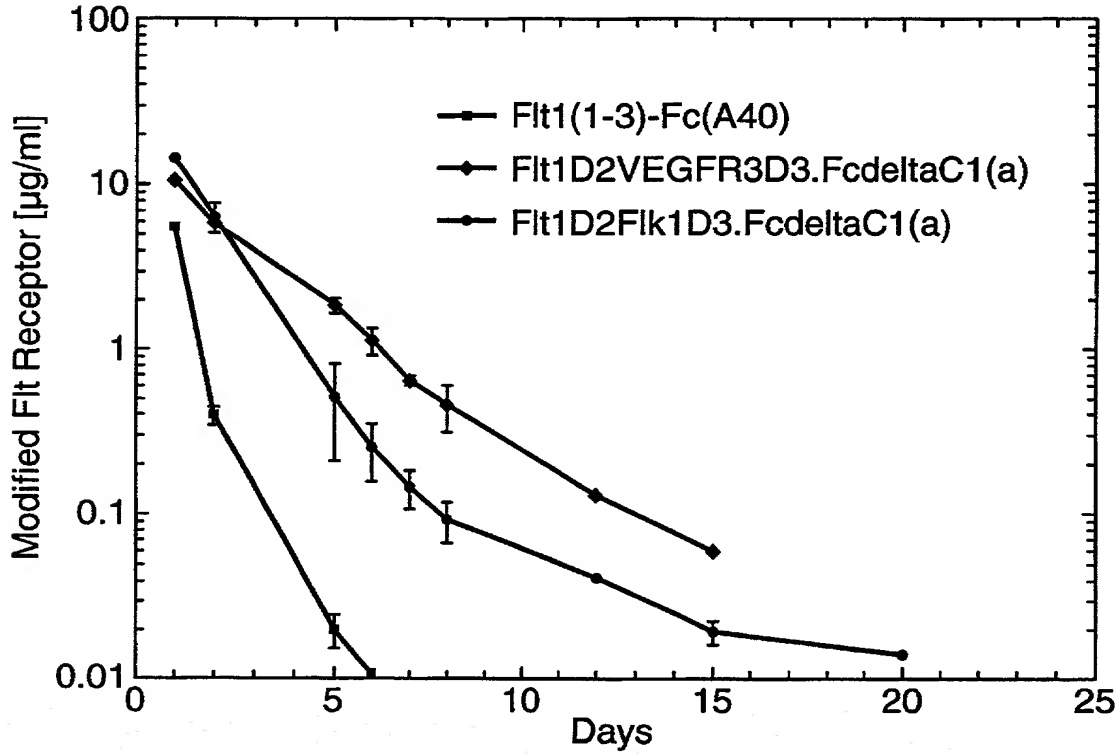

Flt1(1-3)-Fc(A40)
Flt1D2VEGFR3D3.FcdeltaC1(a)
Flt1D2Flk1D3.FcdeltaC1(a)
Modified Flt Receptor [µg/ml]
100
10
1
0.1
0.01
0
5
10
15
20
25
Days

Fig.39.

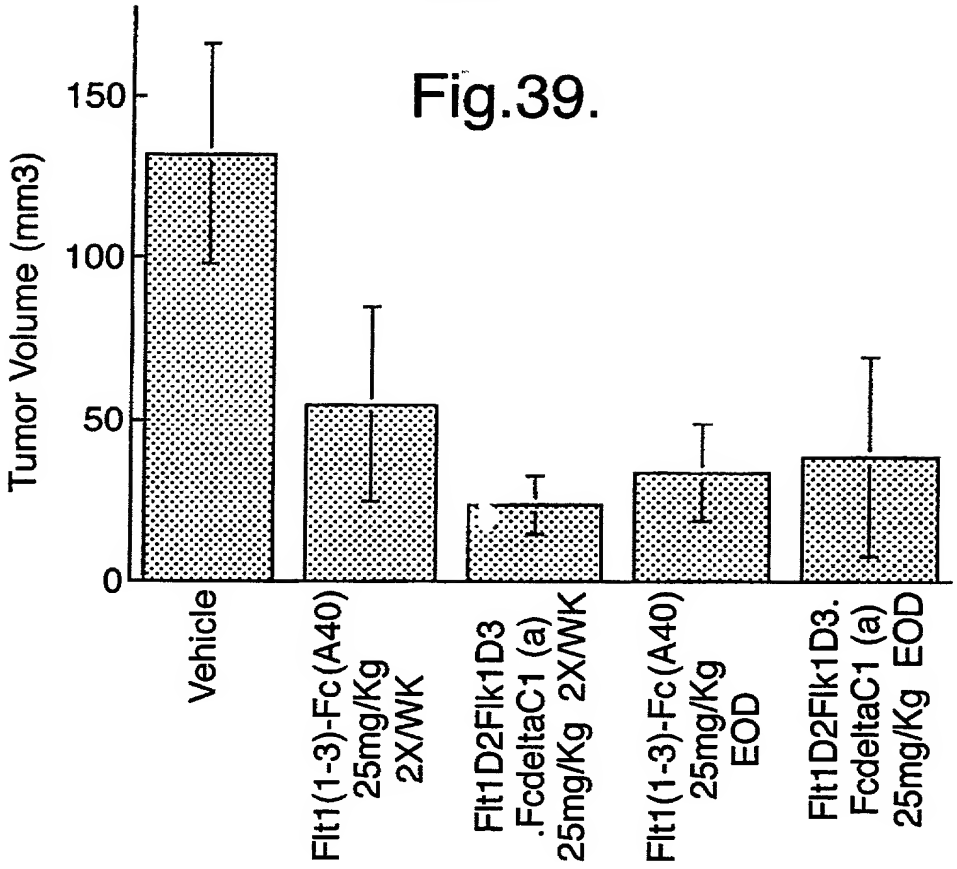

Tumor Volume (mm3)
150
100
50
0
Vehicle
Flt1(1-3)-Fc(A40) 25mg/Kg 2X/WK
Flt1D2Flk1D3 .FcdeltaC1 (a) 25mg/Kg 2X/WK
Flt1(1-3)-Fc(A40) 25mg/Kg EOD
Flt1D2Flk1D3. FcdeltaC1 (a) 25mg/Kg EOD

Fig.40.

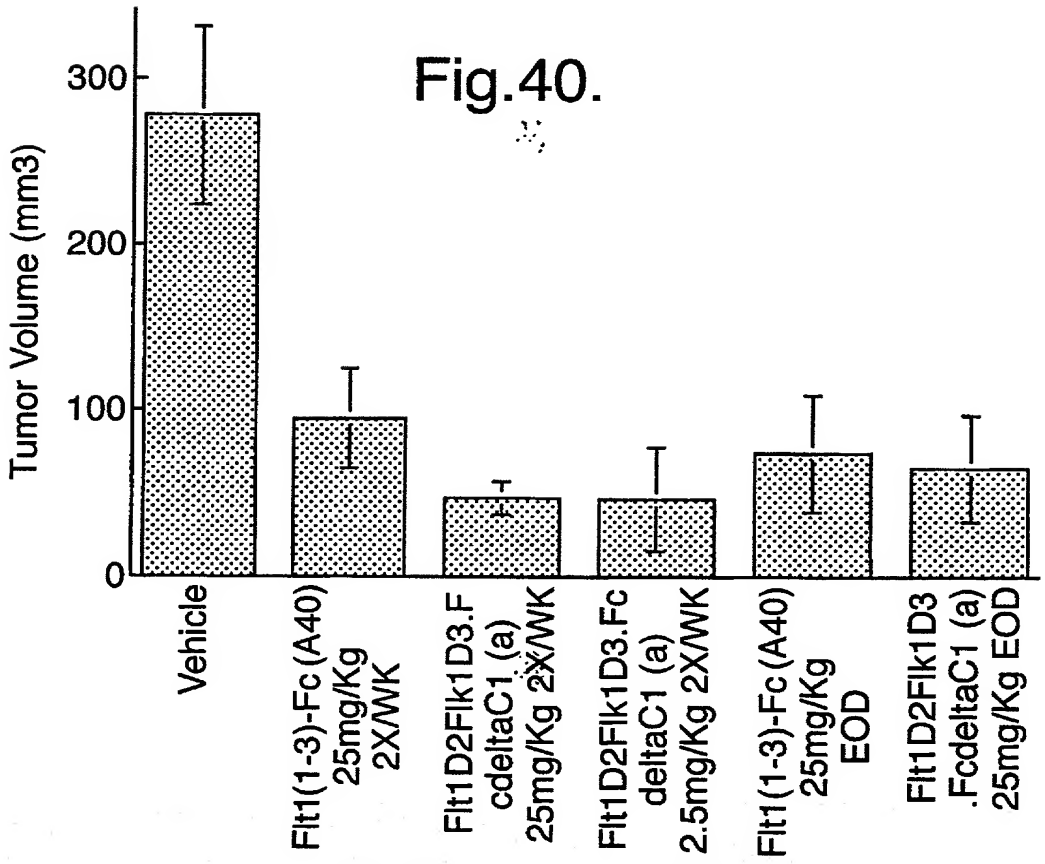

Tumor Volume (mm3)
300
200
100
0
Vehicle
Flt1(1-3)-Fc(A40) 25mg/Kg 2X/WK
Flt1D2Flk1D3.F cdeltaC1 (a) 25mg/Kg 2X/WK
Flt1D2Flk1D3.Fc deltaC1 (a) 2.5mg/Kg 2X/WK
Flt1(1-3)-Fc(A40) 25mg/Kg EOD
Flt1D2Flk1D3 .FcdeltaC1 (a) 25mg/Kg EOD

Fig.41.

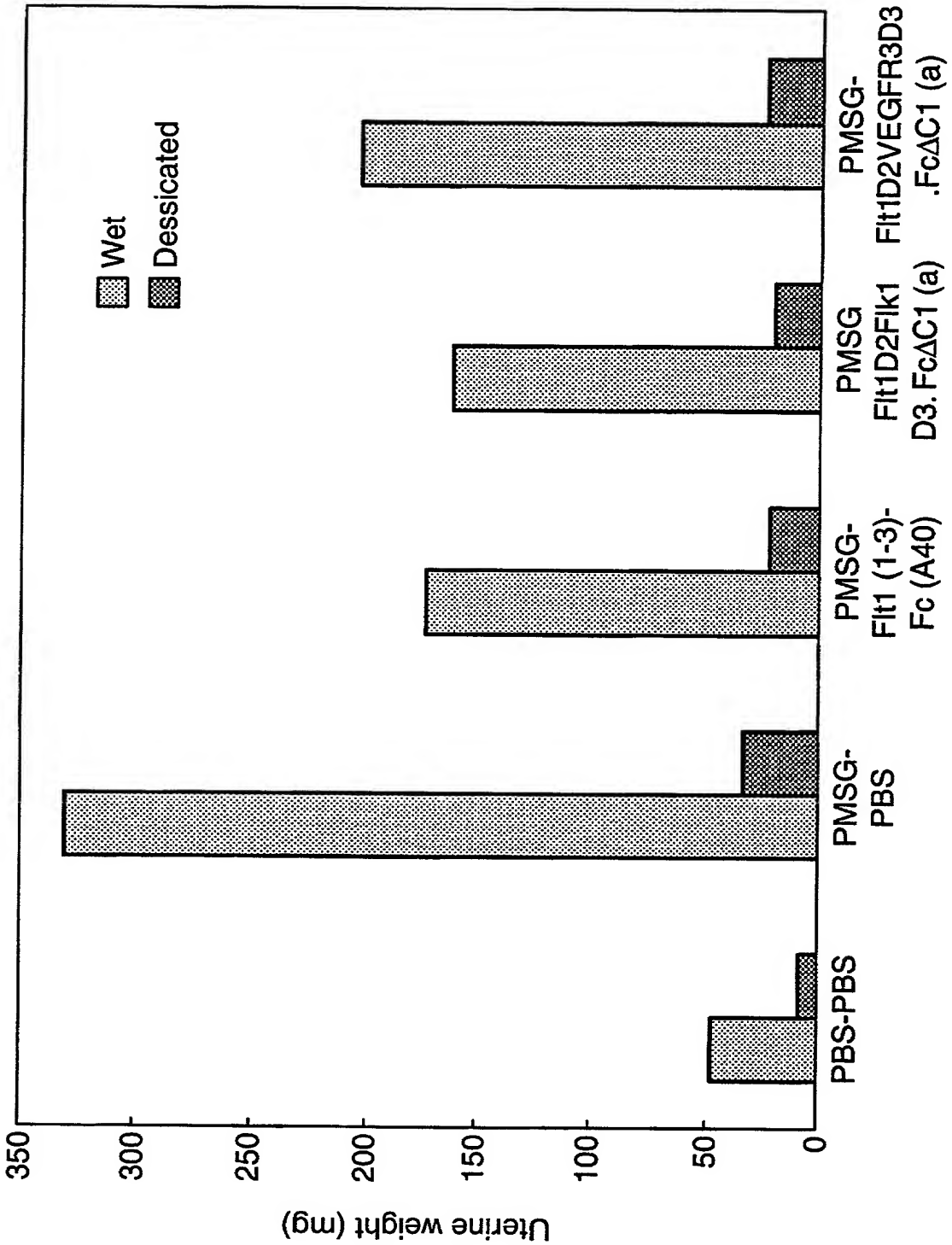

Uterine weight (mg)
350
300
250
200
150
100
50
0
Wet
Dessicated
PBS-PBS
PMSG-PBS
PMSG-Flt1 (1-3)-Fc (A40)
PMSG Flt1D2Flk1 D3.FcΔC1 (a)
PMSG-Flt1D2VEGFR3D3 .FcΔC1 (a)

Fig.42A.

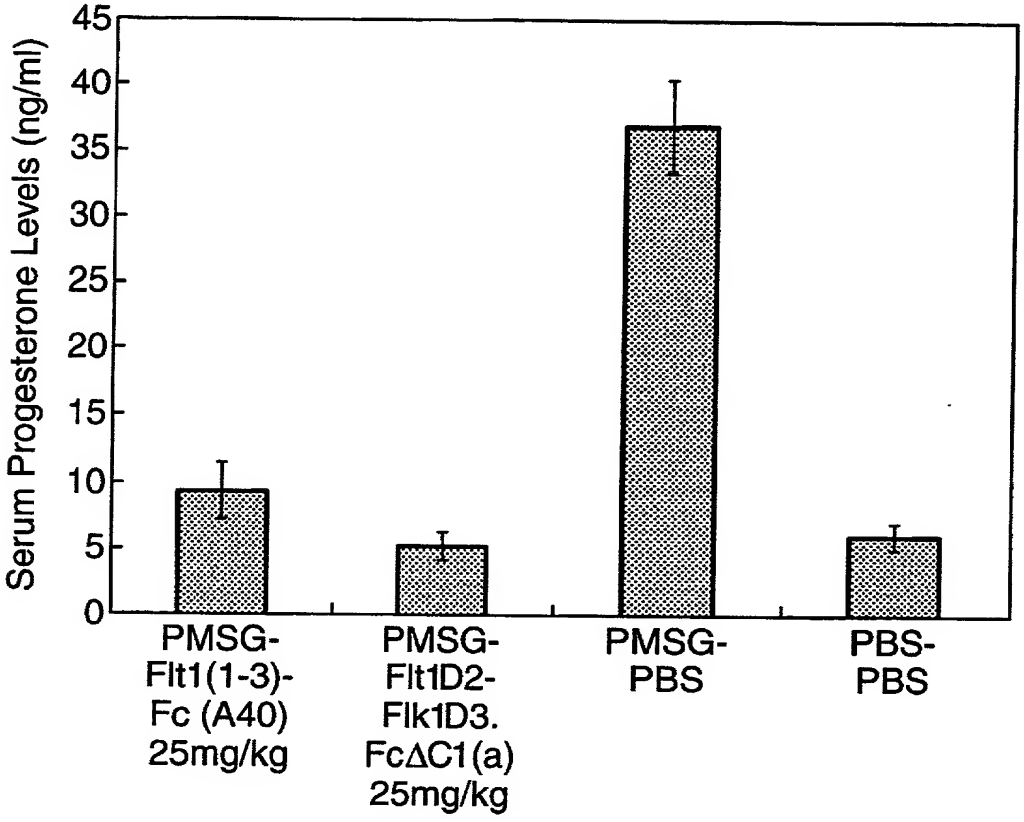
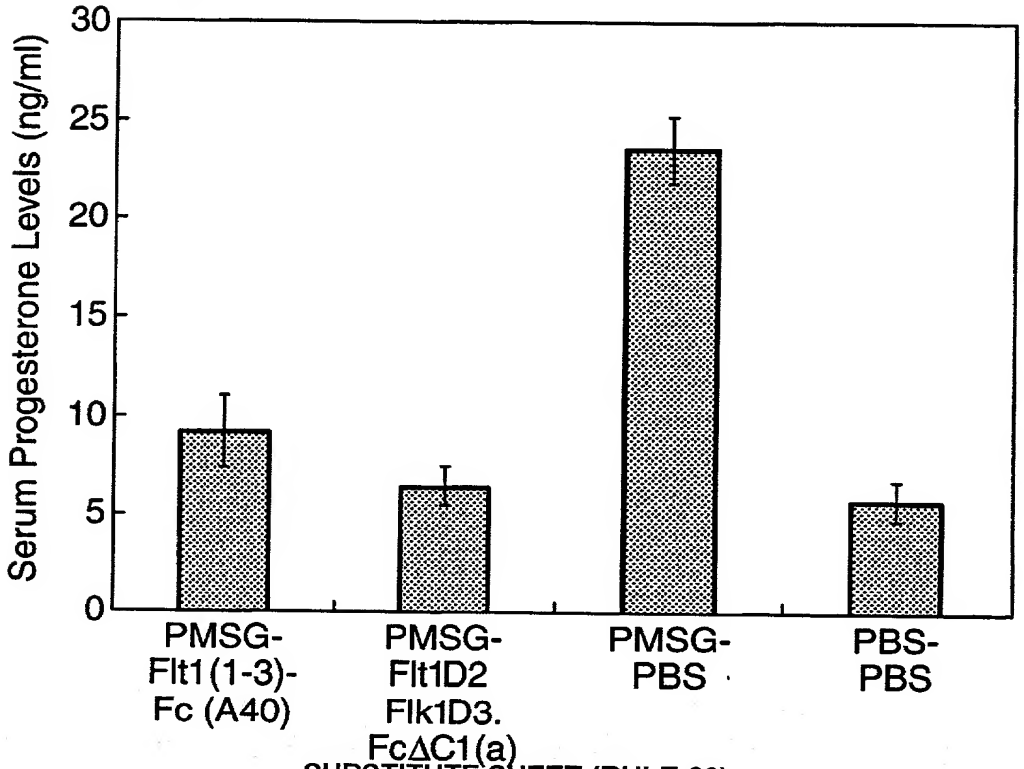


Fig.42B.



DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled **MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES AND METHODS OF MAKING AND USING THEREOF**, which is the United States national stage filing of International Application PCT/US00/14142 filed May 23, 2000.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to in the oath or declaration.

I acknowledge the duty to disclose information of which I am aware that is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PCT/US00/14142 filed May 23, 2000

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States Application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) that occurred between the filing date of the prior application and the national or PCT international filing date of this application:

USSN 60/138,133 filed June 8, 1999

(3)

And I hereby appoint Joseph M. Sorrentino (Registration No. 32,598), Gail M. Kempler (Registration No. 32,143), and Linda O. Palladino (Registration No. 45,636) each of them my attorneys and agent, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International

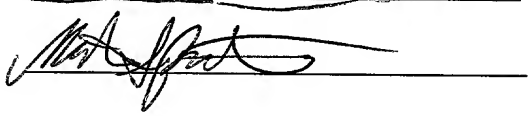
Applications that are based thereon under the provisions of the Patent Cooperation Treaty.

Please address all communications, and direct all telephone calls, regarding this application to:

Linda O. Palladino
Regeneron Pharmaceuticals, Inc.
777 Old Saw Mill River Road
Tarrytown, New York 10591
Tel. (914-345-7400)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Inventor: NICHOLAS J. PAPADOPOULOS

Signature:  Date: 12/06/01

Citizenship: United States of America

Residence: 59 Heritage Lane NY

Lagrangeville, New York 12540

Post Office Address: same as residence

2-00

Inventor: SAMUEL DAVIS

Signature: Samuel Davis Date: 12/6/01

Citizenship: United States of America

Residence: 332 W. 88th Street, #B2 NY
New York, New York 10024

Post Office Address: same as residence

3-00

Inventor: GEORGE D. YANCOPOULOS

Signature: G D Yan Date: 12-6-01

Citizenship: United States of America

Residence: 1519 Baptist Church Road NY
Yorktown Heights, New York 10598

Post Office Address: same as residence

SEQUENCE LISTING

<110> Nicholas J. Papadopoulos et al.

<120> MODIFIED CHIMERIC POLYPEPTIDES WITH IMPROVED PHARMACOKINETIC PROPERTIES AND METHODS OF MAKING AND USING THEREOF

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<140> Not Yet Known
<141> Filed Herewith

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<151> 2000-05-23

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<151> 1999-06-08

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 1               5                  10                  15

tgt ctg ctt ctc aca gga tct agt tca ggt tca aaa tta aaa gat cct       96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
             20                  25                  30

gaa ctg agt tta aaa ggc acc cag cac atc atg caa gca ggc cag aca      144
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
         35                  40                  45

ctg cat ctc caa tgc agg ggg gaa gca gcc cat aaa tgg tct ttg cct      192
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
     50                  55                  60

gaa atg gtg agt aag gaa agc gaa agg ctg agc ata act aaa tct gcc      240
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
 65                  70                  75                  80

tgt gga aga aat ggc aaa caa ttc tgc agt act tta acc ttg aac aca      288
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
                 85                  90                  95

gct caa gca aac cac act ggc ttc tac agc tgc aaa tat cta gct gta      336
Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
            100                 105                 110
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cct act tca aag aag aag gaa aca gaa tct gca atc tat ata ttt att      384
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
        115                 120                 125

agt gat aca ggt aga cct ttc gta gag atg tac agt gaa atc ccc gaa      432
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
    130                 135                 140

att ata cac atg act gaa gga agg gag ctc gtc att ccc tgc cgg gtt      480
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145                 150                 155                 160

acg tca cct aac atc act gtt act tta aaa aag ttt cca ctt gac act      528
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
                165                 170                 175

ttg atc cct gat gga aaa cgc ata atc tgg gac agt aga aag ggc ttc      576
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
            180                 185                 190

atc ata tca aat gca acg tac aaa gaa ata ggg ctt ctg acc tgt gaa      624
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
        195                 200                 205

gca aca gtc aat ggg cat ttg tat aag aca aac tat ctc aca cat cga      672
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
    210                 215                 220

caa acc aat aca atc ata gat gtc caa ata agc aca cca cgc cca gtc      720
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225                 230                 235                 240

aaa tta ctt aga ggc cat act ctt gtc ctc aat tgt act gct acc act      768
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
                245                 250                 255

ccc ttg aac acg aga gtt caa atg acc tgg agt tac cct gat gaa aaa      816
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys
            260                 265                 270

aat aag aga gct tcc gta agg cga cga att gac caa agc aat tcc cat      864
Asn Lys Arg Ala Ser Val Arg Arg Arg Ile Asp Gln Ser Asn Ser His
        275                 280                 285

gcc aac ata ttc tac agt gtt ctt act att gac aaa atg cag aac aaa      912
Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys
    290                 295                 300

gac aaa gga ctt tat act tgt cgt gta agg agt gga cca tca ttc aaa      960
Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys
305                 310                 315                 320

tct gtt aac acc tca gtg cat ata tat gat aaa gca ggc ccg ggc gag     1008
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu
                325                 330                 335

ccc aaa tct tgt gac aaa act cac aca tgc cca ccg tgc cca gca cct     1056
Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
            340                 345                 350
```

```
gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc cca aaa ccc aag      1104
Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
        355                 360                 365

gac acc ctc atg atc tcc cgg acc cct gag gtc aca tgc gtg gtg gtg      1152
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
    370                 375                 380

gac gtg agc cac gaa gac cct gag gtc aag ttc aac tgg tac gtg gac      1200
Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
385                 390                 395                 400

ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag gag cag tac      1248
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
                405                 410                 415

aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg cac cag gac      1296
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
            420                 425                 430

tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac aaa gcc ctc      1344
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
        435                 440                 445

cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa ggg cag ccc cga      1392
Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
    450                 455                 460

gaa cca cag gtg tac acc ctg ccc cca tcc cgg gat gag ctg acc aag      1440
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
465                 470                 475                 480

aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc tat ccc agc gac      1488
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
                485                 490                 495

atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac aac tac aag      1536
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
            500                 505                 510

acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc ttc ctc tac agc      1584
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
        515                 520                 525

aag ctc acc gtg gac aag agc agg tgg cag cag ggg aac gtc ttc tca      1632
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
    530                 535                 540

tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg cag aag agc      1680
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
545                 550                 555                 560

ctc tcc ctg tct ccg ggt aaa tga                                     1704
Leu Ser Leu Ser Pro Gly Lys
                565
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<210> 2
<211> 567
<212> PRT
<213> Homo sapiens

<400> 2

Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1   5   10   15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
            20   25   30
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
        35   40   45
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
    50   55   60
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
65   70   75   80
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
                85   90   95
Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
            100   105   110
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
        115   120   125
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
    130   135   140
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145   150   155   160
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
                165   170   175
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
            180   185   190
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
        195   200   205
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
    210   215   220
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225   230   235   240
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
                245   250   255
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys
            260   265   270
Asn Lys Arg Ala Ser Val Arg Arg Arg Ile Asp Gln Ser Asn Ser His
        275   280   285
Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys
    290   295   300
Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys
305   310   315   320
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu
                325   330   335
Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
            340   345   350
Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
        355   360   365
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
    370   375   380
Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
385   390   395   400
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
                405   410   415
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
            420   425   430
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
        435   440   445
Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
    450   455   460
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys

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465                 470                 475                 480
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
                485                 490                 495
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
            500                 505                 510
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
        515                 520                 525
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
    530                 535                 540
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
545                 550                 555                 560
Leu Ser Leu Ser Pro Gly Lys
                565
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<210> 3
<211> 1674
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)...(1671)

<400> 3

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atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg ctc agc       48
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15

tgt ctg ctt ctc aca gga tct agt tca ggt tca aaa tta aaa gat cct       96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
             20                  25                  30

gaa ctg agt tta aaa ggc acc cag cac atc atg caa gca ggc cag aca      144
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
         35                  40                  45

ctg cat ctc caa tgc agg ggg gaa gca gcc cat aaa tgg tct ttg cct      192
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
     50                  55                  60

gaa atg gtg agt aag gaa agc gaa agg ctg agc ata act aaa tct gcc      240
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
 65                  70                  75                  80

tgt gga aga aat ggc aaa caa ttc tgc agt act tta acc ttg aac aca      288
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
                 85                  90                  95

gct caa gca aac cac act ggc ttc tac agc tgc aaa tat cta gct gta      336
Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
            100                 105                 110

cct act tca aag aag aag gaa aca gaa tct gca atc tat ata ttt att      384
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
        115                 120                 125

agt gat aca ggt aga cct ttc gta gag atg tac agt gaa atc ccc gaa      432
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
    130                 135                 140

att ata cac atg act gaa gga agg gag ctc gtc att ccc tgc cgg gtt      480
```

```
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145                 150                 155                 160

acg tca cct aac atc act gtt act tta aaa aag ttt cca ctt gac act      528
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
                165                 170                 175

ttg atc cct gat gga aaa cgc ata atc tgg gac agt aga aag ggc ttc      576
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
            180                 185                 190

atc ata tca aat gca acg tac aaa gaa ata ggg ctt ctg acc tgt gaa      624
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
        195                 200                 205

gca aca gtc aat ggg cat ttg tat aag aca aac tat ctc aca cat cga      672
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
    210                 215                 220

caa acc aat aca atc ata gat gtc caa ata agc aca cca cgc cca gtc      720
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225                 230                 235                 240

aaa tta ctt aga ggc cat act ctt gtc ctc aat tgt act gct acc act      768
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
                245                 250                 255

ccc ttg aac acg aga gtt caa atg acc tgg agt tac cct gat gaa att      816
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Ile
            260                 265                 270

gac caa agc aat tcc cat gcc aac ata ttc tac agt gtt ctt act att      864
Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile
        275                 280                 285

gac aaa atg cag aac aaa gac aaa gga ctt tat act tgt cgt gta agg      912
Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg
    290                 295                 300

agt gga cca tca ttc aaa tct gtt aac acc tca gtg cat ata tat gat      960
Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp
305                 310                 315                 320

aaa gca ggc ccg ggc gag ccc aaa tct tgt gac aaa act cac aca tgc     1008
Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys
                325                 330                 335

cca ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc     1056
Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu
            340                 345                 350

ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag     1104
Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu
        355                 360                 365

gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag     1152
Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys
    370                 375                 380

ttc aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag     1200
Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys
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385                 390                 395                 400

ccg cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc     1248
Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu
                405                 410                 415

acc gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag     1296
Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys
            420                 425                 430

gtc tcc aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa     1344
Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
        435                 440                 445

gcc aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc     1392
Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser
    450                 455                 460

cgg gat gag ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa     1440
Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys
465                 470                 475                 480

ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag     1488
Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln
                485                 490                 495

ccg gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc     1536
Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
            500                 505                 510

tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag     1584
Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
        515                 520                 525

cag ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac     1632
Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn
    530                 535                 540

cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa tga             1674
His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
545                 550                 555
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<210> 4
<211> 557
<212> PRT
<213> Homo sapiens

<400> 4

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Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
            20                  25                  30
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
        35                  40                  45
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
    50                  55                  60
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
65                  70                  75                  80
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
                85                  90                  95
```

```
Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
            100                 105                 110
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
        115                 120                 125
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
    130                 135                 140
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145                 150                 155                 160
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
                165                 170                 175
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
            180                 185                 190
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
        195                 200                 205
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
    210                 215                 220
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225                 230                 235                 240
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
                245                 250                 255
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Ile
            260                 265                 270
Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile
        275                 280                 285
Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg
    290                 295                 300
Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr Asp
305                 310                 315                 320
Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys
                325                 330                 335
Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu
            340                 345                 350
Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu
        355                 360                 365
Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys
    370                 375                 380
Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys
385                 390                 395                 400
Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu
                405                 410                 415
Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys
            420                 425                 430
Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
        435                 440                 445
Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser
    450                 455                 460
Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys
465                 470                 475                 480
Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln
                485                 490                 495
Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
            500                 505                 510
Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
        515                 520                 525
Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn
    530                 535                 540
His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
545                 550                 555
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<210> 5
<211> 1359

<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)...(1356)

<400> 5

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Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15

tgt ctg ctt ctc aca gga tct agt tcc gga ggt aga cct ttc gta gag       96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
            20                  25                  30

atg tac agt gaa atc ccc gaa att ata cac atg act gaa gga agg gag      144
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
        35                  40                  45

ctc gtc att ccc tgc cgg gtt acg tca cct aac atc act gtt act tta      192
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
    50                  55                  60

aaa aag ttt cca ctt gac act ttg atc cct gat gga aaa cgc ata atc      240
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65                  70                  75                  80

tgg gac agt aga aag ggc ttc atc ata tca aat gca acg tac aaa gaa      288
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
                85                  90                  95

ata ggg ctt ctg acc tgt gaa gca aca gtc aat ggg cat ttg tat aag      336
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
            100                 105                 110

aca aac tat ctc aca cat cga caa acc aat aca atc ata gat gtc caa      384
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln
        115                 120                 125

ata agc aca cca cgc cca gtc aaa tta ctt aga ggc cat act ctt gtc      432
Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly His Thr Leu Val
    130                 135                 140

ctc aat tgt act gct acc act ccc ttg aac acg aga gtt caa atg acc      480
Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr
145                 150                 155                 160

tgg agt tac cct gat gaa att gac caa agc aat tcc cat gcc aac ata      528
Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn Ile
                165                 170                 175

ttc tac agt gtt ctt act att gac aaa atg cag aac aaa gac aaa gga      576
Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys Asp Lys Gly
            180                 185                 190

ctt tat act tgt cgt gta agg agt gga cca tca ttc aaa tct gtt aac      624
Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn
        195                 200                 205

acc tca gtg cat ata tat gat aaa gca ggc ccg ggc gag ccc aaa tct      672
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Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser
    210                 215                 220

tgt gac aaa act cac aca tgc cca ccg tgc cca gca cct gaa ctc ctg      720
Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

ggg gga ccg tca gtc ttc ctc ttc ccc cca aaa ccc aag gac acc ctc      768
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

atg atc tcc cgg acc cct gag gtc aca tgc gtg gtg gtg gac gtg agc      816
Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

cac gaa gac cct gag gtc aag ttc aac tgg tac gtg gac ggc gtg gag      864
His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

gtg cat aat gcc aag aca aag ccg cgg gag gag cag tac aac agc acg      912
Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
    290                 295                 300

tac cgt gtg gtc agc gtc ctc acc gtc ctg cac cag gac tgg ctg aat      960
Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

ggc aag gag tac aag tgc aag gtc tcc aac aaa gcc ctc cca gcc ccc     1008
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

atc gag aaa acc atc tcc aaa gcc aaa ggg cag ccc cga gaa cca cag     1056
Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

gtg tac acc ctg ccc cca tcc cgg gat gag ctg acc aag aac cag gtc     1104
Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val
        355                 360                 365

agc ctg acc tgc ctg gtc aaa ggc ttc tat ccc agc gac atc gcc gtg     1152
Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

gag tgg gag agc aat ggg cag ccg gag aac aac tac aag acc acg cct     1200
Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

ccc gtg ctg gac tcc gac ggc tcc ttc ttc ctc tac agc aag ctc acc     1248
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

gtg gac aag agc agg tgg cag cag ggg aac gtc ttc tca tgc tcc gtg     1296
Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

atg cat gag gct ctg cac aac cac tac acg cag aag agc ctc tcc ctg     1344
Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

tct ccg ggt aaa tga                                                1359
Ser Pro Gly Lys
```

450

<210> 6
<211> 452
<212> PRT
<213> Homo sapiens

<400> 6
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1   5   10   15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
            20   25   30
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
        35   40   45
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
    50   55   60
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65   70   75   80
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
                85   90   95
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
            100   105   110
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln
        115   120   125
Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly His Thr Leu Val
    130   135   140
Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr
145   150   155   160
Trp Ser Tyr Pro Asp Glu Ile Asp Gln Ser Asn Ser His Ala Asn Ile
                165   170   175
Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys Asp Lys Gly
            180   185   190
Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys Ser Val Asn
        195   200   205
Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser
    210   215   220
Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225   230   235   240
Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245   250   255
Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260   265   270
His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275   280   285
Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
    290   295   300
Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305   310   315   320
Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325   330   335
Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340   345   350
Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val
        355   360   365
Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370   375   380
Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385   390   395   400
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405   410   415

```
Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430
Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445
Ser Pro Gly Lys
    450
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<210> 7
<211> 1389
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)...(1386)

<400> 7

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atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg ctc agc      48
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15

tgt ctg ctt ctc aca gga tct agt tcc gga ggt aga cct ttc gta gag      96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
            20                  25                  30

atg tac agt gaa atc ccc gaa att ata cac atg act gaa gga agg gag     144
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
        35                  40                  45

ctc gtc att ccc tgc cgg gtt acg tca cct aac atc act gtt act tta     192
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
    50                  55                  60

aaa aag ttt cca ctt gac act ttg atc cct gat gga aaa cgc ata atc     240
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65                  70                  75                  80

tgg gac agt aga aag ggc ttc atc ata tca aat gca acg tac aaa gaa     288
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
                85                  90                  95

ata ggg ctt ctg acc tgt gaa gca aca gtc aat ggg cat ttg tat aag     336
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
            100                 105                 110

aca aac tat ctc aca cat cga caa acc aat aca atc ata gat gtc caa     384
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln
        115                 120                 125

ata agc aca cca cgc cca gtc aaa tta ctt aga ggc cat act ctt gtc     432
Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly His Thr Leu Val
    130                 135                 140

ctc aat tgt act gct acc act ccc ttg aac acg aga gtt caa atg acc     480
Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr
145                 150                 155                 160

tgg agt tac cct gat gaa aaa aat aag aga gct tcc gta agg cga cga     528
Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg Arg
                165                 170                 175
```

```
att gac caa agc aat tcc cat gcc aac ata ttc tac agt gtt ctt act      576
Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr
            180                 185                 190

att gac aaa atg cag aac aaa gac aaa gga ctt tat act tgt cgt gta      624
Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val
        195                 200                 205

agg agt gga cca tca ttc aaa tct gtt aac acc tca gtg cat ata tat      672
Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr
    210                 215                 220

gat aaa gca ggc ccg ggc gag ccc aaa tct tgt gac aaa act cac aca      720
Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230                 235                 240

tgc cca ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc      768
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
                245                 250                 255

ctc ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct      816
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
            260                 265                 270

gag gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc      864
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
        275                 280                 285

aag ttc aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca      912
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
    290                 295                 300

aag ccg cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc      960
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
305                 310                 315                 320

ctc acc gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc     1008
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
                325                 330                 335

aag gtc tcc aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc     1056
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
            340                 345                 350

aaa gcc aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca     1104
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
        355                 360                 365

tcc cgg gat gag ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc     1152
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
    370                 375                 380

aaa ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg     1200
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
385                 390                 395                 400

cag ccg gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac     1248
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
                405                 410                 415

ggc tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg     1296
```

```
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
            420                 425                 430

cag cag ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac     1344
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
        435                 440                 445

aac cac tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa             1386
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    450                 455                 460

tga                                                                 1389
```

<210> 8
<211> 462
<212> PRT
<213> Homo sapiens

<400> 8

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Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
            20                  25                  30
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
        35                  40                  45
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
    50                  55                  60
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65                  70                  75                  80
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
                85                  90                  95
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
            100                 105                 110
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Gln
        115                 120                 125
Ile Ser Thr Pro Arg Pro Val Lys Leu Leu Arg Gly His Thr Leu Val
    130                 135                 140
Leu Asn Cys Thr Ala Thr Thr Pro Leu Asn Thr Arg Val Gln Met Thr
145                 150                 155                 160
Trp Ser Tyr Pro Asp Glu Lys Asn Lys Arg Ala Ser Val Arg Arg Arg
                165                 170                 175
Ile Asp Gln Ser Asn Ser His Ala Asn Ile Phe Tyr Ser Val Leu Thr
            180                 185                 190
Ile Asp Lys Met Gln Asn Lys Asp Lys Gly Leu Tyr Thr Cys Arg Val
        195                 200                 205
Arg Ser Gly Pro Ser Phe Lys Ser Val Asn Thr Ser Val His Ile Tyr
    210                 215                 220
Asp Lys Ala Gly Pro Gly Glu Pro Lys Ser Cys Asp Lys Thr His Thr
225                 230                 235                 240
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
                245                 250                 255
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
            260                 265                 270
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
        275                 280                 285
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
    290                 295                 300
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
305                 310                 315                 320
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
                325                 330                 335
```

```
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
            340                 345                 350
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
        355                 360                 365
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
    370                 375                 380
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
385                 390                 395                 400
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
                405                 410                 415
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
            420                 425                 430
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
        435                 440                 445
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    450                 455                 460
```

<210> 9
<211> 1704
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)...(1701)

<400> 9

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atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg ctc agc        48
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15

tgt ctg ctt ctc aca gga tct agt tca ggt tca aaa tta aaa gat cct        96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
            20                  25                  30

gaa ctg agt tta aaa ggc acc cag cac atc atg caa gca ggc cag aca       144
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
        35                  40                  45

ctg cat ctc caa tgc agg ggg gaa gca gcc cat aaa tgg tct ttg cct       192
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
    50                  55                  60

gaa atg gtg agt aag gaa agc gaa agg ctg agc ata act aaa tct gcc       240
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
65                  70                  75                  80

tgt gga aga aat ggc aaa caa ttc tgc agt act tta acc ttg aac aca       288
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
                85                  90                  95

gct caa gca aac cac act ggc ttc tac agc tgc aaa tat cta gct gta       336
Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
            100                 105                 110

cct act tca aag aag aag gaa aca gaa tct gca atc tat ata ttt att       384
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
        115                 120                 125

agt gat aca ggt aga cct ttc gta gag atg tac agt gaa atc ccc gaa       432
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
```

```
    130                 135                 140

att ata cac atg act gaa gga agg gag ctc gtc att ccc tgc cgg gtt      480
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145                 150                 155                 160

acg tca cct aac atc act gtt act tta aaa aag ttt cca ctt gac act      528
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
                165                 170                 175

ttg atc cct gat gga aaa cgc ata atc tgg gac agt aga aag ggc ttc      576
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
            180                 185                 190

atc ata tca aat gca acg tac aaa gaa ata ggg ctt ctg acc tgt gaa      624
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
        195                 200                 205

gca aca gtc aat ggg cat ttg tat aag aca aac tat ctc aca cat cga      672
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
    210                 215                 220

caa acc aat aca atc ata gat gtc caa ata agc aca cca cgc cca gtc      720
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225                 230                 235                 240

aaa tta ctt aga ggc cat act ctt gtc ctc aat tgt act gct acc act      768
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
                245                 250                 255

ccc ttg aac acg aga gtt caa atg acc tgg agt tac cct gat gaa aaa      816
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys
            260                 265                 270

aat aag aac gct tcc gta agg cga cga att gac caa agc aat tcc cat      864
Asn Lys Asn Ala Ser Val Arg Arg Arg Ile Asp Gln Ser Asn Ser His
        275                 280                 285

gcc aac ata ttc tac agt gtt ctt act att gac aaa atg cag aac aaa      912
Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys
    290                 295                 300

gac aaa gga ctt tat act tgt cgt gta agg agt gga cca tca ttc aaa      960
Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys
305                 310                 315                 320

tct gtt aac acc tca gtg cat ata tat gat aaa gca ggc ccg ggc gag     1008
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu
                325                 330                 335

ccc aaa tct tgt gac aaa act cac aca tgc cca ccg tgc cca gca cct     1056
Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
            340                 345                 350

gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc cca aaa ccc aag     1104
Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
        355                 360                 365

gac acc ctc atg atc tcc cgg acc cct gag gtc aca tgc gtg gtg gtg     1152
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
    370                 375                 380
```

```
gac gtg agc cac gaa gac cct gag gtc aag ttc aac tgg tac gtg gac     1200
Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
385                 390                 395                 400

ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag gag cag tac     1248
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
                405                 410                 415

aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg cac cag gac     1296
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
            420                 425                 430

tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac aaa gcc ctc     1344
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
        435                 440                 445

cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa ggg cag ccc cga     1392
Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
    450                 455                 460

gaa cca cag gtg tac acc ctg ccc cca tcc cgg gat gag ctg acc aag     1440
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
465                 470                 475                 480

aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc tat ccc agc gac     1488
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
                485                 490                 495

atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac aac tac aag     1536
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
            500                 505                 510

acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc ttc ctc tac agc     1584
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
        515                 520                 525

aag ctc acc gtg gac aag agc agg tgg cag cag ggg aac gtc ttc tca     1632
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
    530                 535                 540

tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg cag aag agc     1680
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
545                 550                 555                 560

ctc tcc ctg tct ccg ggt aaa tga                                    1704
Leu Ser Leu Ser Pro Gly Lys
                565
```

<210> 10
<211> 567
<212> PRT
<213> Homo sapiens

<400> 10

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Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Lys Leu Lys Asp Pro
            20                  25                  30
Glu Leu Ser Leu Lys Gly Thr Gln His Ile Met Gln Ala Gly Gln Thr
```

35 40 45
Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
50 55 60
Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
65 70 75 80
Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
85 90 95
Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
100 105 110
Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
115 120 125
Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
130 135 140
Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
145 150 155 160
Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
165 170 175
Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
180 185 190
Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
195 200 205
Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
210 215 220
Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
225 230 235 240
Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
245 250 255
Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys
260 265 270
Asn Lys Asn Ala Ser Val Arg Arg Arg Ile Asp Gln Ser Asn Ser His
275 280 285
Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys
290 295 300
Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys
305 310 315 320
Ser Val Asn Thr Ser Val His Ile Tyr Asp Lys Ala Gly Pro Gly Glu
325 330 335
Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
340 345 350
Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
355 360 365
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
370 375 380
Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
385 390 395 400
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
405 410 415
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
420 425 430
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
435 440 445
Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
450 455 460
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
465 470 475 480
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
485 490 495
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
500 505 510
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
515 520 525

```
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
    530                 535                 540
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
545                 550                 555                 560
Leu Ser Leu Ser Pro Gly Lys
                565
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<210> 11
<211> 1453
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (69)...(1442)

<400> 11

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aagcttgggc tgcaggtcga tcgactctag aggatcgatc cccgggcgag ctcgaattcg      60
caaccacc atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg      110
         Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu
          1               5                  10

ctc agc tgt ctg ctt ctc aca gga tct agt tcc gga ggt aga cct ttc      158
Leu Ser Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe
 15                  20                  25                  30

gta gag atg tac agt gaa atc ccc gaa att ata cac atg act gaa gga      206
Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly
                 35                  40                  45

agg gag ctc gtc att ccc tgc cgg gtt acg tca cct aac atc act gtt      254
Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val
             50                  55                  60

act tta aaa aag ttt cca ctt gac act ttg atc cct gat gga aaa cgc      302
Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg
         65                  70                  75

ata atc tgg gac agt aga aag ggc ttc atc ata tca aat gca acg tac      350
Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr
     80                  85                  90

aaa gaa ata ggg ctt ctg acc tgt gaa gca aca gtc aat ggg cat ttg      398
Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu
 95                  100                 105                 110

tat aag aca aac tat ctc aca cat cga caa acc aat aca atc ata gat      446
Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp
                115                 120                 125

gtg gtt ctg agt ccg tct cat gga att gaa cta tct gtt gga gaa aag      494
Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu Lys
            130                 135                 140

ctt gtc tta aat tgt aca gca aga act gaa cta aat gtg ggg att gac      542
Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile Asp
        145                 150                 155

ttc aac tgg gaa tac cct tct tcg aag cat cag cat aag aaa ctt gta      590
Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu Val
    160                 165                 170
```

```
aac cga gac cta aaa acc cag tct ggg agt gag atg aag aaa ttt ttg      638
Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe Leu
175                 180                 185                 190

agc acc tta act ata gat ggt gta acc cgg agt gac caa gga ttg tac      686
Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu Tyr
                195                 200                 205

acc tgt gca gca tcc agt ggg ctg atg acc aag aag aac agc aca ttt      734
Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr Phe
            210                 215                 220

gtc agg gtc cat gaa aag ggc ccg ggc gac aaa act cac aca tgc cca      782
Val Arg Val His Glu Lys Gly Pro Gly Asp Lys Thr His Thr Cys Pro
        225                 230                 235

ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc      830
Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe
    240                 245                 250

ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc      878
Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val
255                 260                 265                 270

aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc      926
Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe
                275                 280                 285

aac tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg      974
Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro
            290                 295                 300

cgg gag gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc     1022
Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr
        305                 310                 315

gtc ctg cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc     1070
Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
    320                 325                 330

tcc aac aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc     1118
Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala
335                 340                 345                 350

aaa ggg cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg     1166
Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg
                355                 360                 365

gat gag ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc     1214
Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly
            370                 375                 380

ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg     1262
Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
        385                 390                 395

gag aac aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc     1310
Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
    400                 405                 410
```

ttc ttc ctc tat agc aag ctc acc gtg gac aag agc agg tgg cag cag 1358
Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln
415 420 425 430

ggg aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac 1406
Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
435 440 445

tac acg cag aag agc ctc tcc ctg tct ccg ggt aaa tgagcggccg 1452
Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
450 455

c 1453

<210> 12
<211> 458
<212> PRT
<213> Homo sapiens

<400> 12
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
1 5 10 15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
20 25 30
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
35 40 45
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
50 55 60
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65 70 75 80
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
85 90 95
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
100 105 110
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Val Val
115 120 125
Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu Lys Leu Val
130 135 140
Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile Asp Phe Asn
145 150 155 160
Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu Val Asn Arg
165 170 175
Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe Leu Ser Thr
180 185 190
Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu Tyr Thr Cys
195 200 205
Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr Phe Val Arg
210 215 220
Val His Glu Lys Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys
225 230 235 240
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
245 250 255
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
260 265 270
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
275 280 285
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
290 295 300
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
305 310 315 320
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn

```
                325                 330                 335
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
            340                 345                 350
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
        355                 360                 365
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
    370                 375                 380
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
385                 390                 395                 400
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
                405                 410                 415
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
            420                 425                 430
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
        435                 440                 445
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    450                 455
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<210> 13
<211> 1444
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (69)...(1433)

<400> 13

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aagcttgggc tgcaggtcga tcgactctag aggatcgatc cccgggcgag ctcgaattcg     60
caaccacc atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg     110
         Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu
          1               5                  10

ctc agc tgt ctg ctt ctc aca gga tct agt tcc gga ggt aga cct ttc      158
Leu Ser Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe
 15                  20                  25                  30

gta gag atg tac agt gaa atc ccc gaa att ata cac atg act gaa gga      206
Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly
                35                  40                  45

agg gag ctc gtc att ccc tgc cgg gtt acg tca cct aac atc act gtt      254
Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val
            50                  55                  60

act tta aaa aag ttt cca ctt gac act ttg atc cct gat gga aaa cgc      302
Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg
        65                  70                  75

ata atc tgg gac agt aga aag ggc ttc atc ata tca aat gca acg tac      350
Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr
    80                  85                  90

aaa gaa ata ggg ctt ctg acc tgt gaa gca aca gtc aat ggg cat ttg      398
Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu
 95                  100                 105                 110

tat aag aca aac tat ctc aca cat cga caa acc aat aca atc ata gat      446
Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp
                115                 120                 125
```

```
atc cag ctg ttg ccc agg aag tcg ctg gag ctg ctg gta ggg gag aag      494
Ile Gln Leu Leu Pro Arg Lys Ser Leu Glu Leu Leu Val Gly Glu Lys
            130                 135                 140

ctg gtc ctc aac tgc acc gtg tgg gct gag ttt aac tca ggt gtc acc      542
Leu Val Leu Asn Cys Thr Val Trp Ala Glu Phe Asn Ser Gly Val Thr
        145                 150                 155

ttt gac tgg gac tac cca ggg aag cag gca gag cgg ggt aag tgg gtg      590
Phe Asp Trp Asp Tyr Pro Gly Lys Gln Ala Glu Arg Gly Lys Trp Val
    160                 165                 170

ccc gag cga cgc tcc caa cag acc cac aca gaa ctc tcc agc atc ctg      638
Pro Glu Arg Arg Ser Gln Gln Thr His Thr Glu Leu Ser Ser Ile Leu
175                 180                 185                 190

acc atc cac aac gtc agc cag cac gac ctg ggc tcg tat gtg tgc aag      686
Thr Ile His Asn Val Ser Gln His Asp Leu Gly Ser Tyr Val Cys Lys
                195                 200                 205

gcc aac aac ggc atc cag cga ttt cgg gag agc acc gag gtc att gtg      734
Ala Asn Asn Gly Ile Gln Arg Phe Arg Glu Ser Thr Glu Val Ile Val
            210                 215                 220

cat gaa aat ggc ccg ggc gac aaa act cac aca tgc cca ccg tgc cca      782
His Glu Asn Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys Pro
        225                 230                 235

gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc cca aaa      830
Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys
    240                 245                 250

ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc aca tgc gtg      878
Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val
255                 260                 265                 270

gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac tgg tac      926
Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr
                275                 280                 285

gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag gag      974
Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu
            290                 295                 300

cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg cac     1022
Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His
        305                 310                 315

cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac aaa     1070
Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys
    320                 325                 330

gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa ggg cag     1118
Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln
335                 340                 345                 350

ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg gat gag ctg     1166
Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu
                355                 360                 365

acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc tat ccc     1214
```

```
Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro
            370                 375                 380

agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac aac     1262
Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn
        385                 390                 395

tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc ttc ctc     1310
Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu
    400                 405                 410

tat agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg aac gtc     1358
Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val
415                 420                 425                 430

ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg cag     1406
Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln
                435                 440                 445

aag agc ctc tcc ctg tct ccg ggt aaa tgagcggccg c                   1444
Lys Ser Leu Ser Leu Ser Pro Gly Lys
            450                 455


      <210> 14
      <211> 455
      <212> PRT
      <213> Homo sapiens

      <400> 14
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Gly Arg Pro Phe Val Glu
            20                  25                  30
Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu
        35                  40                  45
Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu
    50                  55                  60
Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile
65                  70                  75                  80
Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu
                85                  90                  95
Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys
            100                 105                 110
Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile Asp Ile Gln
        115                 120                 125
Leu Leu Pro Arg Lys Ser Leu Glu Leu Leu Val Gly Glu Lys Leu Val
    130                 135                 140
Leu Asn Cys Thr Val Trp Ala Glu Phe Asn Ser Gly Val Thr Phe Asp
145                 150                 155                 160
Trp Asp Tyr Pro Gly Lys Gln Ala Glu Arg Gly Lys Trp Val Pro Glu
                165                 170                 175
Arg Arg Ser Gln Gln Thr His Thr Glu Leu Ser Ser Ile Leu Thr Ile
            180                 185                 190
His Asn Val Ser Gln His Asp Leu Gly Ser Tyr Val Cys Lys Ala Asn
        195                 200                 205
Asn Gly Ile Gln Arg Phe Arg Glu Ser Thr Glu Val Ile Val His Glu
    210                 215                 220
Asn Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro
225                 230                 235                 240
Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys
```

```
                245                 250                 255
Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val
            260                 265                 270
Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp
        275                 280                 285
Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr
    290                 295                 300
Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp
305                 310                 315                 320
Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu
                325                 330                 335
Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg
            340                 345                 350
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
        355                 360                 365
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
    370                 375                 380
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
385                 390                 395                 400
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
                405                 410                 415
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
            420                 425                 430
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
        435                 440                 445
Leu Ser Leu Ser Pro Gly Lys
    450                 455
```

<210> 15
<211> 1377
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)...(1374)

<400> 15

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atg gtc agc tac tgg gac acc ggg gtc ctg ctg tgc gcg ctg ctc agc      48
Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15

tgt ctg ctt ctc aca gga tct agt tcc gga agt gat acc ggt aga cct      96
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Asp Thr Gly Arg Pro
            20                  25                  30

ttc gta gag atg tac agt gaa atc ccc gaa att ata cac atg act gaa     144
Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu
        35                  40                  45

gga agg gag ctc gtc att ccc tgc cgg gtt acg tca cct aac atc act     192
Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr
    50                  55                  60

gtt act tta aaa aag ttt cca ctt gac act ttg atc cct gat gga aaa     240
Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys
65                  70                  75                  80

cgc ata atc tgg gac agt aga aag ggc ttc atc ata tca aat gca acg     288
Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr
                85                  90                  95
```

```
tac aaa gaa ata ggg ctt ctg acc tgt gaa gca aca gtc aat ggg cat      336
Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His
            100                 105                 110

ttg tat aag aca aac tat ctc aca cat cga caa acc aat aca atc ata      384
Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile
        115                 120                 125

gat gtg gtt ctg agt ccg tct cat gga att gaa cta tct gtt gga gaa      432
Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu
    130                 135                 140

aag ctt gtc tta aat tgt aca gca aga act gaa cta aat gtg ggg att      480
Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile
145                 150                 155                 160

gac ttc aac tgg gaa tac cct tct tcg aag cat cag cat aag aaa ctt      528
Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu
                165                 170                 175

gta aac cga gac cta aaa acc cag tct ggg agt gag atg aag aaa ttt      576
Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe
            180                 185                 190

ttg agc acc tta act ata gat ggt gta acc cgg agt gac caa gga ttg      624
Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu
        195                 200                 205

tac acc tgt gca gca tcc agt ggg ctg atg acc aag aag aac agc aca      672
Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr
    210                 215                 220

ttt gtc agg gtc cat gaa aag gac aaa act cac aca tgc cca ccg tgc      720
Phe Val Arg Val His Glu Lys Asp Lys Thr His Thr Cys Pro Pro Cys
225                 230                 235                 240

cca gca cct gaa ctc ctg ggg gga ccg tca gtc ttc ctc ttc ccc cca      768
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
                245                 250                 255

aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag gtc aca tgc      816
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
            260                 265                 270

gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac tgg      864
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
        275                 280                 285

tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag      912
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
    290                 295                 300

gag cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg      960
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
305                 310                 315                 320

cac cag gac tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac     1008
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
                325                 330                 335
```

```
aaa gcc ctc cca gcc ccc atc gag aaa acc atc tcc aaa gcc aaa ggg     1056
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
            340                 345                 350

cag ccc cga gaa cca cag gtg tac acc ctg ccc cca tcc cgg gat gag     1104
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
        355                 360                 365

ctg acc aag aac cag gtc agc ctg acc tgc ctg gtc aaa ggc ttc tat     1152
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
    370                 375                 380

ccc agc gac atc gcc gtg gag tgg gag agc aat ggg cag ccg gag aac     1200
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
385                 390                 395                 400

aac tac aag acc acg cct ccc gtg ctg gac tcc gac ggc tcc ttc ttc     1248
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
                405                 410                 415

ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg aac     1296
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
            420                 425                 430

gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg     1344
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
        435                 440                 445

cag aag agc ctc tcc ctg tct ccg ggt aaa tga                        1377
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    450                 455
```

<210> 16
<211> 458
<212> PRT
<213> Homo sapiens

<400> 16

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Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15
Cys Leu Leu Leu Thr Gly Ser Ser Ser Gly Ser Asp Thr Gly Arg Pro
            20                  25                  30
Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His Met Thr Glu
        35                  40                  45
Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro Asn Ile Thr
    50                  55                  60
Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys
65                  70                  75                  80
Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr
                85                  90                  95
Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val Asn Gly His
            100                 105                 110
Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn Thr Ile Ile
        115                 120                 125
Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu
    130                 135                 140
Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile
145                 150                 155                 160
Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu
                165                 170                 175
```

Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe
180 185 190
Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu
195 200 205
Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr
210 215 220
Phe Val Arg Val His Glu Lys Asp Lys Thr His Thr Cys Pro Pro Cys
225 230 235 240
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
245 250 255
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
260 265 270
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
275 280 285
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
290 295 300
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
305 310 315 320
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
325 330 335
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
340 345 350
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
355 360 365
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
370 375 380
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
385 390 395 400
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
405 410 415
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
420 425 430
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
435 440 445
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
450 455

<210> 17
<211> 430
<212> PRT
<213> Homo sapiens

<400> 17
Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu Ile Ile His
1 5 10 15
Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val Thr Ser Pro
20 25 30
Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr Leu Ile Pro
35 40 45
Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe Ile Ile Ser
50 55 60
Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu Ala Thr Val
65 70 75 80
Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg Gln Thr Asn
85 90 95
Thr Ile Ile Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser
100 105 110
Val Gly Glu Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn
115 120 125
Val Gly Ile Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His
130 135 140

Lys Lys Leu Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met
145 150 155 160
Lys Lys Phe Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp
165 170 175
Gln Gly Leu Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys
180 185 190
Asn Ser Thr Phe Val Arg Val His Glu Lys Gly Pro Gly Asp Lys Thr
195 200 205
His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser
210 215 220
Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg
225 230 235 240
Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro
245 250 255
Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala
260 265 270
Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val
275 280 285
Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr
290 295 300
Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr
305 310 315 320
Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
325 330 335
Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys
340 345 350
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
355 360 365
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
370 375 380
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser
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